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The Fine Structure of the Chorio-Allantoic Membrane of the Normal Chick Embryo: A Control Study for Virus Work.
EMIL BORYSKO AND FREDERIK B. BANG 257

The Development of the Virus of Newcastle Disease in Epithelial and Fibroblast Cells in Tissue Culture. FREDERIK B. BANG 291

The Development of Newcastle Disease Virus in Cells of the Chorio-Allantoic Membrane as Studied by Thin Sections.
FREDERIK B. BANG 309

Splenic-Portal Venography. A Technique Utilizing Percutaneous Injection of Radiopaque Material into the Spleen.
HENRY T. BAHNSON, ROBERT D. SLOAN AND ALFRED BLALOCK 331

Book Reviews 346

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THE FINE STRUCTURE OF THE CHORIO-ALLANTOIC MEMBRANE OF THE NORMAL CHICK EMBRYO

A Control Study for Virus Work[1]

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The utilization of the chorio-allantoic membrane of the chick embryo for the growth of virus was first reported by Woodruff and Goodpasture in 1931 (1) and, since that time, it has become one of the most widely used tissues for the study and propagation of viruses in the laboratory. With the development of techniques for the examination of tissues in the electron microscope, this membrane has also become quite popular for the study of the submicroscopic aspects of viral infections. Its convenient position in the egg and its extreme thinness enable it to be rapidly prepared for electron microscopy with a minimum of damage to the cells. This work deals with the normal morphology of the cells of the chorio-allantoic membrane as seen in the electron microscope and is intended as a basis for the study of virus infections of the same tissue (2, 3).

EXPERIMENTAL TECHNIQUES

In any morphological study of fixed and sectioned material, the problem of fixation has always been of paramount importance (4). This is particularly true of tissues studied in the electron microscope (5). In the course of this study, several fixatives were tried, including acetic alcohol, cold acetone, neutral formalin, 2 per cent osmic acid in distilled water and a 1 per cent osmic acid solution buffered to pH 7.3–7.4 with a veronal acetate buffer (5). Preparations were also made by means of the freeze-drying technique. In agreement with the work of others (5, 6, 7, 8, 9) osmic acid fixation preserved best the general appearance and structural details as compared with the living cell observed under the phase contrast microscope (Figs. 5, 6, 7, 8). It produced a fine precipitate throughout the cell with hardly any distortion of the cellular structures. Fixation in neutral formalin followed by embedding in methacrylate also produced a fine precipitate but the mitochondria and lipoid droplets were destroyed or badly distorted (Fig. 3). The degree of distortion with this procedure was most marked in the red cells, many of which were swollen to several times their original diameters (Fig. 27). The other methods of fixation resulted in a coarse coagulation of the protoplasm, destroying or obscuring structures to the extent that the preparations were useless for study in the electron microscope (Figs. 1, 2, 4).

In order to determine the optimum fixation time, a number of small specimens were taken from the chorio-allantoic membrane of an 11 day chick embryo and fixed for periods of time ranging from 10 minutes to 9 hours. A definite decrease in the finely precipitated material

[1] This investigation was supported by a research grant from the National Institutes of Health, Public Health Service, Bethesda, Maryland.

[2] Damon Runyon Memorial Fund Fellow.

became apparent after nine hours of exposure to the fixative. This was interpreted to mean that osmic acid slowly dissolves or otherwise removes the fine precipitate in the fixed tissues. Exposure to osmic acid for from 10 minutes to 4 hours produced no apparent change in the fixation image. Fixation time was therefore subsequently kept within these limits. The fixation image produced by 1 per cent buffered osmic acid was not significantly different from that produced by the unbuffered 2 per cent osmic acid solution, although this was not true for other tissues that have been studied in our laboratory.

In the early stages of this work and in previous work (10, 11) samples of the chorio-allantoic membrane were obtained by allowing it to drop away from the shell membrane and then cutting out a piece about 1 cm. square and placing it in fixative contained in a vial. In the process of separating the chorio-allantoic membrane from the shell membrane, the cells which had been lying against the shell membrane were frequently damaged and had an alveolar appearance (Fig. 31) resulting from damage to the cell membrane similar to that described by Chambers (12) for other types of cells. To avoid this, the porosity of the shell membrane (13) was taken advantage of and fixation was accomplished by flooding an area of the shell membrane with osmic acid while the chorio-allantoic membrane was still attached to it. After a few minutes, the cells of the chorio-allantoic membrane were sufficiently well fixed so that the fibrous shell membrane could be easily separated with a minimum of damage to the underlying cells. The effectiveness of this method of fixation was demonstrated by the rapid blackening of the blood in the vessels of the mesodermal layer and the lack of shrinkage or distortion of the membrane. Specimens of the membrane so obtained were quite stiff and flat when removed from the egg, which simplified subsequent handling and orientation. After removal from the embryo, the membrane was placed in the osmic acid solution for the remainder of the fixation period.

The fixed membranes were dehydrated in a series of increasing concentrations of ethyl alcohol and embedded in a mixture of n-butyl and methyl methacrylate by the methods of Newman *et al.* (14). Partial polymerization of the methacrylate to a highly viscous state before immersion of the tissue made it possible to orient the membranes so that they could be sectioned in a plane perpendicular to their surfaces, the viscosity of the embedding mass serving to hold the tissues in proper position during the final stages of polymerization (15). Sectioning was done on a Spencer 820 microtome, using the thermal expansion of a brass block to advance the specimen by fractions of a micron. The sections were cut with steel microtome knives sharpened on a flat leather strop charged with diamond powder. The methacrylate embedding matrix was removed from the sections in acetone and they were re-embedded in a thin collodion film and mounted on stainless steel screens for examination in the electron microscope. The sections were not allowed to dry in air at any time in this process. A comparative study of section quality showed that no artifacts were produced by the replacement of methacrylate with collodion. Sections mounted in collodion were superior in clarity and contrast to those mounted in methacrylate. The sections were not shadowed.

The observations reported here were made on a total of 47 membranes taken from normal chick embryos ranging from 11 to 17 days of age. The embryos were taken directly from a Jamesway commerical incubator which maintained optimum humidity at 39–40°C. and which automatically turned the eggs every two hours. All eggs were purchased as fertile white leghorn eggs from a local farm. Of the membranes studied, 35 were fixed in osmic acid, 5 in neutral formalin, 3 in acetic alcohol, 1 in cold acetone and 3 were frozen dried preparations. An RCA EMU-2C electron microscope was used exclusively.

OBSERVATIONS

The chorio-allantoic membrane is formed by the fusion of the allantois and the chorion which begins at about the fifth day of incubation (16, 17). Struc-

turally, it is composed of three distinct layers (Fig. 6). The innermost layer of epithelial cells, lining the allantoic cavity, is usually one cell in thickness though areas may be found where there is an overlapping of two and occasionally three cells. This layer will be referred to as the allantoic layer. The middle layer is composed of areolar connective tissue in which the blood vessels supplying the membrane are contained. This layer will be referred to as the mesodermal layer. The outermost layer, lying in close contact with the shell membrane, generally consists of several layers of epithelial cells and numerous capillaries. Since it was derived from the chorion (with the exception of the capillaries), it will be referred to as the chorionic layer.

The Allantoic Layer

We have considered the cells of the allantoic layer more useful than the cells of the other two layers for the study of cellular pathology because of their relatively uniform structure. The cells comprising this layer are bounded by the allantoic fluid, the adjacent cells and the areolar connective tissue of the mesodermal layer (Fig. 7). Each boundary has a distinct appearance and will be considered separately. The cell surface in contact with the allantoic fluid was consistently characterized by the presence of variable numbers of small projections (Figs. 7, 8) which we have named "microvilli"[3] to distinguish them from other types of surface projections (cilia, pseudopods, brush borders, blebs, etc.). These microvilli appeared as club-shaped projections extending into the allantoic sac. They were approximately 0.1 micron in width, ranging from 0.1 to 1.0 micron in length (Figs. 12, 15).

In whole mounts of cells from this layer which were grown as primary explants in roller tube tissue culture, entire microvilli could be seen (Fig. 13). In one of these preparations, the average length of a microvillus was found to be about 0.30 micron. No special internal structure was detected; they merely appeared as simple projections of the cytoplasm. The number of microvilli on any single cell as seen both on cells grown in tissue culture and in sections, varied considerably. Frequently, a cell was seen which had a great many covering its entire surface while its neighboring cells had only three or four or, in rare instances, none at all. In order to get some idea of the number of microvilli that project from the surface of a cell, a section was selected in which they appeared to be particularly abundant and the number of microvilli counted for a distance of 75 microns. Four cells were included in this span. Assuming that the section was 0.1 micron in thickness and that the cells had a plane surface area of 225 square microns in contact with the allantoic fluid, it was calculated that there were about 3,600 microvilli per cell in the area from which the section was taken. Assuming the microvilli to be cylinders 0.1 micron in diameter and 1.0 micron in length, their total surface area was calculated to

[3] They have been referred to briefly in previous publications (11, 18).

be approximately 1,135 square microns. This is roughly five times the surface area of a cell devoid of microvilli.

Though microvilli were occasionally seen in clumps associated with dense cytoplasmic material (Fig. 15) and sometimes seemed particularly numerous and uniformly distributed over the entire surface of individual cells, as seen in Fig. 14, no correlation could be found between the number of microvilli on a cell and the number of mitochondria or other cytoplasmic components within the same cell. Microvilli were present in specimens of the membrane taken from beneath the air sac, the central region and the narrow end of the egg. A comparison of specimens from three embryos that were incubated for 11 days under standard conditions with three embryos which were similarly incubated except that the periodic turning of the eggs was eliminated showed no difference in the number or appearance of the microvilli. Similarly, the microvilli on cells grown in roller tube culture with constant motion were no different from those grown in Maximow depression slides without motion.

Structures resembling microvilli have been found in several other tissues which we have examined in our laboratory and have been seen by workers in other laboratories (19, 20). They usually appeared on epithelial cells lining liquid-filled sacs or tubes.

The cells of the allantoic layer were generally not separated from each other by a distinct cell membrane, the cytoplasm of adjacent cells appearing, at first glance, to be continuous. Frequently, however, rows of vacuoles containing small amounts of fine granular material seemed to mark the cell boundaries. In the strands of cytoplasm separating the vacuoles large, roughly rectangular bodies were often seen (Fig. 7). These bodies were approximately 0.2 microns in width and of variable length. Similar dense bodies were seen irrespective of the presence of intercellular vacuoles and they were often found at the free surface of the cell, where they sometimes protruded into the allantoic sac (Fig. 8). Underexposed prints of the negatives revealed that these dense bodies consisted of symmetrical condensations of material on the plasma membranes of neighboring cells (Figs. 9, 10, 11). The plasma membranes appeared as two fine parallel lines within the dense material, each approximately 0.01 micron in thickness, separated by a space about 0.02 micron in width. These "complementary granules" are reminiscent of the bodies found within the intercellular bridges of the stratum spinulosum of the skin. In addition to the intercellular vacuoles and complementary granules described above, indistinct labyrinthine areas were also seen at the boundaries between the cells.

The cell surface in contact with the mesodermal layer appeared to have a simple structure in the sense that specialized projections, vacuoles or granules were lacking. It generally appeared either as a dense fine line or as a thicker and less well defined boundary. In a few sections the surface membrane appeared to be double, consisting of two fine parallel lines, each approximately 0.05 micron in thickness (Fig. 16).

The fine precipitate seen in the cytoplasm of the cells of the allantoic layer after fixation with osmic acid consisted of particles ranging in size from 0.1 to about 0.01 micron. They varied in shape from dense granules to fine filaments and appeared to be distributed at random throughout the cell. There was no evidence, in any of the preparations, of a peripheral zone of ectoplasm morphologically distinguishable from the cytoplasm. Occasionally, hollow filamentous structures about 0.1 micron in diameter were seen in the cytoplasm (Fig. 16). They were quite convoluted, sometimes showing a tendency to branch. That these structures were hollow tubes was evidenced by the rounded appearance of their cut ends. In many respects they resembled the "endoplasmic reticulum" seen by Porter and Palade in other cells (5, 21). Whether these structures are the same as the intracellular membranes or filaments (5, 19, 22, 23) seen in glandular and kidney cells is as yet undetermined.

The mitochondria in these cells were usually elongated bodies which appeared round in cross section. Some of these bodies had a well-defined limiting membrane and a complex internal structure, resembling the mitochondria seen in other tissues (23, 24), but some appeared to be structureless (Figs. 17, 20). In the plane of the sections, the number of mitochondria in a single cell varied from one to more than fifteen.

Roughly spherical masses of dense, non-granular material ranging from 0.2 to 2.0 microns in diameter, were usually present in the cytoplasm (Fig. 17). We have interpreted these to be lipoid droplets because of their extreme density after fixation with osmic acid and because of their apparent absence in formalin fixed material. In some specimens, they were more numerous in the region adjacent to the free surface of the cell. Intracellular vacuoles containing variable amounts of finely granular material were frequently seen in the cytoplasm. They seemed to be randomly distributed throughout the cytoplasm in some cells but, in others they showed a tendency to be more numerous in the portion of the cell next to the allantoic cavity (Fig. 19).

Nuclei in the cells of the allantoic layer were usually round or oval in cross-section (Fig. 7). Frequently, however, nuclei were seen which had indentations or fissures extending to within a fraction of a micron of the opposite side of the nucleus (Fig. 8). Despite the fact that many of the cells in this layer were in some phase of mitotic division, as could be seen in preparations fixed and stained for optical microscopy, we were not able to identify mitotic figures in the electron microscope preparations. It is possible that osmic acid fails to preserve the mitotic apparatus or that the mitotic apparatus is preserved in such a manner that it cannot be recognized in thin sections. It is also possible that chromosomes may be indistinguishable, in thin sections, from mitochondria or other cytoplasmic organelles after the disappearance of the nuclear membrane.

The nuclear membrane usually appeared as a dense structure of variable

thickness but, occasionally, it seemed to be composed of two distinct, sharply defined layers (25). It was interesting to note that when the nuclear membranes appeared to be doubled, there was a corresponding doubling of the membrane in contact with the mesodermal layer and the membranes surrounding the intercellular vacuoles (Fig. 16).

Although the majority of the nucleoli were homogeneous, finely granular bodies, many had an internal structure consisting of large dense granules or filaments which has been described elsewhere (26, 27). The nucleoli exhibited a variety of rounded shapes and were frequently seen to be attached to the nuclear membrane.

The precipitated material of the nucleoplasm had the same general appearance as that of the cytoplasm. There seemed to be a tendency for the fine granules and filaments to aggregate loosely, forming shapeless dense areas in the nucleus and giving it a mottled appearance. In a number of nuclei, large masses of dense material were found lying close to the nuclear membrane. In some cases, these masses were flattened against the membrane, giving the impression of localized increases in the thickness of the nuclear membrane. These may represent the chromocenters seen by Fell and Hughes in fixed and living cells (28).

The Mesodermal Layer

The thickness of this layer seemed to vary with the diameter of the blood vessels in any particular region of the membrane. It was very loosely organized, consisting of widely separated, stellate fibroblasts, blood vessels and lymphatics lying in a loose open meshwork of unbranched fibrils. The fibrils were uniformly about 0.03 micron in thickness. In some preparations, the fibrils showed a periodicity of about 0.06 micron suggesting a resemblance to collagen. Additional evidence of their similarity to collagen was provided by digesting fresh specimens of the membrane with a dilute solution of trypsin in buffered neutral saline. The fibrous mass remaining after this treatment was washed with distilled water and dried on film-covered screens. In the electron microscope, the fibers were seemingly of indefinite length, possessing all of the fine structural characteristics of collagen.

The fibroblasts were characterized by their long, branching cytoplasmic projections extending in all directions away from the nucleus (Fig. 21). The nuclear region formed the thickest portion of the cell, though the nucleus itself was surrounded by a relatively thin layer of cytoplasm. The cell boundaries of the fibroblasts were simple in comparison with the boundaries of the cells of the allantoic layer. The plasma membrane was usually indistinct, though it often appeared as a thin, dense line in some regions of the cell surface.

The cytoplasm of the fibroblasts, as well as the mitochondria, lipoid droplets and intracellular vacuoles were similar to those found in the cells of the allan-

toic layer. In addition to these structures, long filaments, occasionally branched and apparently hollow, were seen (Fig. 22). They were about 0.1 micron in width with a wall thickness of about 0.02–0.03 micron. In some sections, they measured as much as 20 microns in length. If these elements were, in fact, hollow, round filaments, it is highly improbable that we could have obtained sections in which such lengths appeared since their thickness approximated the thickness of the sections. Segments of contorted filaments with the above dimensions lying in the plane of a section would rarely exceed a few microns in length. We are inclined to believe that they are actually intracellular ribbons. The lack of any suggestion of tubular structure in the cut ends of the filaments supports this interpretation. In the chorio-allantoic membrane, the intracellular ribbons were found only in the fibroblasts, making one wonder whether they were in some way associated with the production of collagan. No collagen-like fibrils were seen within the cells. Except for fibroblasts in the walls of blood vessels, the collagen fibrils appeared to be randomly oriented with respect to the surfaces of the fibroblasts.

The general architecture of the walls of the arteries, capillaries, veins and lymphatics in the mesodermal layer varied from simple membranes composed of a single layer of endothelial cells to complex structures consisting of an endothelial lining surrounded by one or more layers of fibroblasts and of smooth muscle cells. Though the endothelial cells were quite diverse in shape, the fine structure of their cytoplasmic and nuclear constituents was similar to that of the cells of the allantoic layer (Fig. 23). The cell boundaries, however, were delineated by simple membranes, lacking the specialized structures seen in the allantoic layer. In a few endothelial cells in the lining of the blood vessels, the nuclei were abnormal in appearance, often showing a striking resemblance to the nuclei of immature red blood cells (Figs. 26, 27). It is probable that they represent early stages in the development of red blood corpuscles from endothelial cells (29).

The Chorionic Layer

This layer is unusual in that it represents a condition in which mesodermal tissue has come to lie outside of ectodermal tissue. In the early stages of development, the mesoderm is completely covered by epithelial cells derived from the ectoderm of the chorion. At about the 10th day, blood vessels of the mesoderm begin to penetrate the chorionic layer and form an extensive anastomosing capillary plexus on its outer surface in direct contact with the shell membrane (16). The capillary walls were seen to be about 0.2 microns in thickness (Figs. 28, 30). The presence of mitochondria in the capillary walls indicates that these walls are functional cytoplasmic extensions of the endothelial cells.

The epithelial cells of the chorionic layer were flat to cuboidal in shape (Figs. 29, 30). Many contained large masses of material similar to red blood

cells in density and texture, creating the impression that erythrocytes were being phagocytized (Fig. 30). The fine structure of the epithelial cells was so diversified that a complete description will not be attempted here. The normal variations in structure and the changes induced by the separation of the chorio-allantoic membrane from the shell membrane make a study of the effect of viruses more difficult with this layer of cells.

CONCLUSIONS

In the course of this work, it became clear that in studies of infected cells, normal microvilli must be differentiated from virus filaments, normal inter- and intracellular vacuoles from pathological collections of fluid and normal cytoplasmic inclusions from virus particles. In addition, the necessity of comparing the effect of a variety of fixatives with each other and with the appearance of the living cells as seen in the phase contrast microscope was emphasized (30). One cannot adequately study the relationship of viruses to mitochondria (31) or to the red blood cells using formalin fixation and methacrylate embedding, since this procedure destroys the mitochondria and distorts the erythrocytes.

The degree of cellular disruption brought about by separating the chorio-allantoic membrane from the shell membrane before fixation was surprising. It suggests that this type of cell exposure, which is so commonly used to study the growth of pox viruses, may produce variations in the susceptibility of the cells to the virus which do not occur when other types of inoculation are used (32).

SUMMARY

1. A study of the fine structure of the chorio-allantoic membrane of the normal chick embryo with the electron microscope is presented.

2. Osmic acid fixation for one-half to four hours best preserved the structures apparent in living cells and gave fewer demonstrable artifacts than other fixatives. Continued exposure of the tissue to osmic acid caused a clearing of the cytoplasm due to an apparent removal of the more finely precipitated substance.

3. Fixation by formalin followed by methacrylate embedding caused distortion of red cells and destruction of the mitochondria.

4. The separation of the chorio-allantoic membrane from the shell membrane in the living embryo was accompanied by damage to the superficial cells and ballooning distortion of some of the cell constituents. This was prevented by fixation of the chorio-allantoic membrane with osmic acid before separating it from the shell membrane.

5. Prominent among a variety of minute structures studied were: (a) Microvilli—Small, finger-like projections which were consistently seen on the free

surface of the epithelial cells lining the allantoic cavity. These were about 0.1 micron in width and varied in length from 0.1 to 1.0 micron. It was estimated that one cell might have as many as 3,600 projecting from its surface. (b) Complementary granules—Dense granules lying between the cells of the allantoic layer which, in reality, consisted of symmetrical condensations on the plasma membranes of neighboring cells. (c) Intracellular "ribbons"—Dense, hollow structures about 0.1 micron in width and up to 20.0 microns in length which were found only in the fibroblasts of the mesodermal layer. (d) Mitochondria—The structure of the mitochondria usually consisted of an external membrane surrounding a complex series of folds and indentations. Variations in structure were noted. (e) Collagen—Extracellular fibers in the mesodermal layer having the appearance and dimensions of collagen. These were seen both in sections and in isolates obtained by tryptic digestion of the membranes. In sections, the fibrils appeared to be randomly oriented with respect to the surfaces of the fibroblasts.

6. The endothelial cells lining the blood vessels of the mesoderm and the walls of the capillaries of the chorionic layer were often so thin that their complete extent could be seen only with the electron microscope.

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PLATES

PLATE I

A comparison of the appearance of cells of the allantoic layer fixed by several different fixation procedures.

Unless otherwise noted, the unit distance on the photographs represents one micron.

FIG. 1. Freeze-drying technique. The alveolar appearance of the entire cell was the usual image obtained, though an occasional cell could be found which was relatively undamaged. The Swiss-cheese appearance was not objectionably apparent in thicker sections which were stained and examined in the optical microscope. Magnification: 12,000×

FIG. 2. Cold acetone fixation. Coarse coagulation of the protoplasm with practically complete destruction of fine structure. Magnification: 12,000×

FIG. 3. Neutral formalin fixation. Fine precipitation of the protoplasm. Nuclear and plasma membranes distinct. Structure of the nucleolus well-preserved. Mitochondria and fat droplets absent. Magnification: 12,000×

FIG. 4. Acetic alcohol fixation. Distortion caused by this fixative was so marked that it was difficult to distinguish the nucleus from the cytoplasm.

It is likely that the distortion produced by these fixatives is more apparent in the electron microscope than in thicker, stained sections examined in the optical microscope because of the lack of a staining differential and the thinness of the sections. It is also possible that artifacts are produced by the methacrylate embedding process which are not produced by paraffin embedding. Magnification: 12,000×

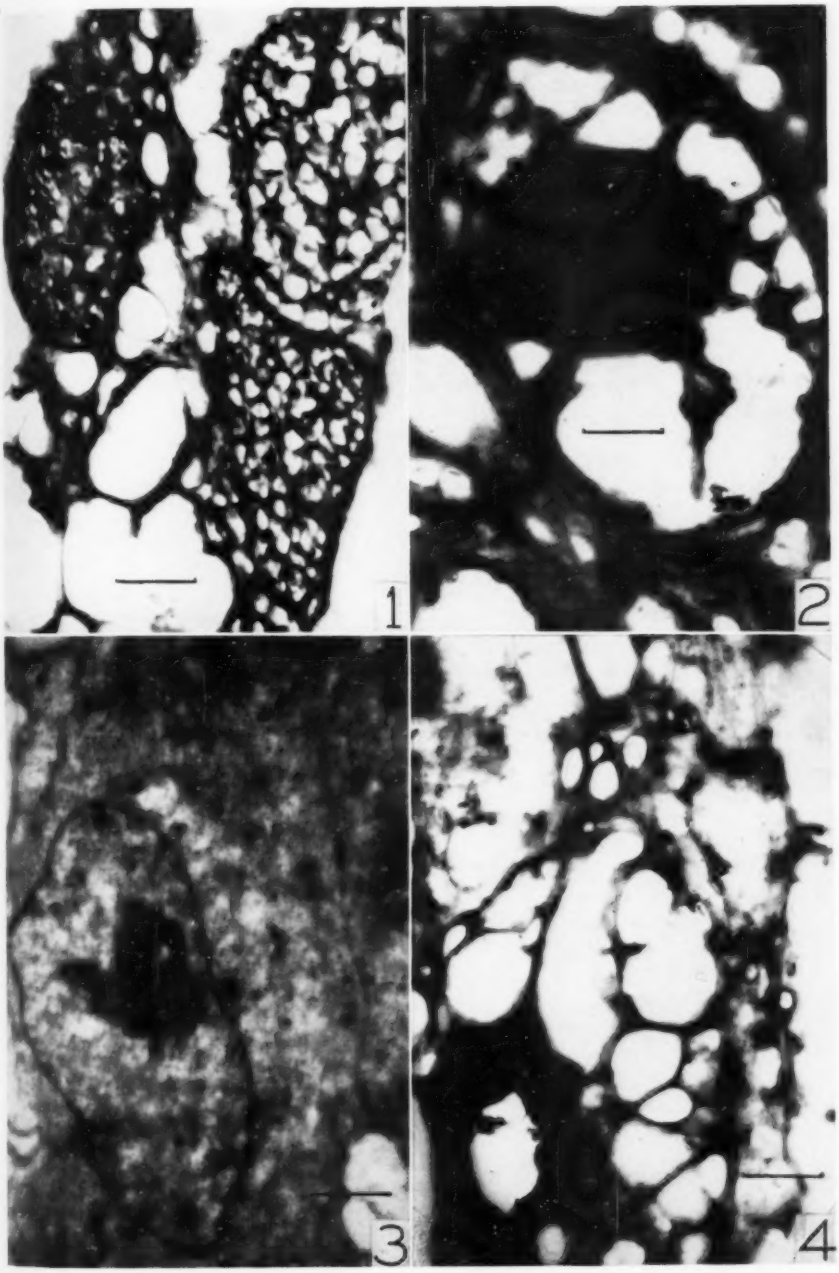
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PLATE II

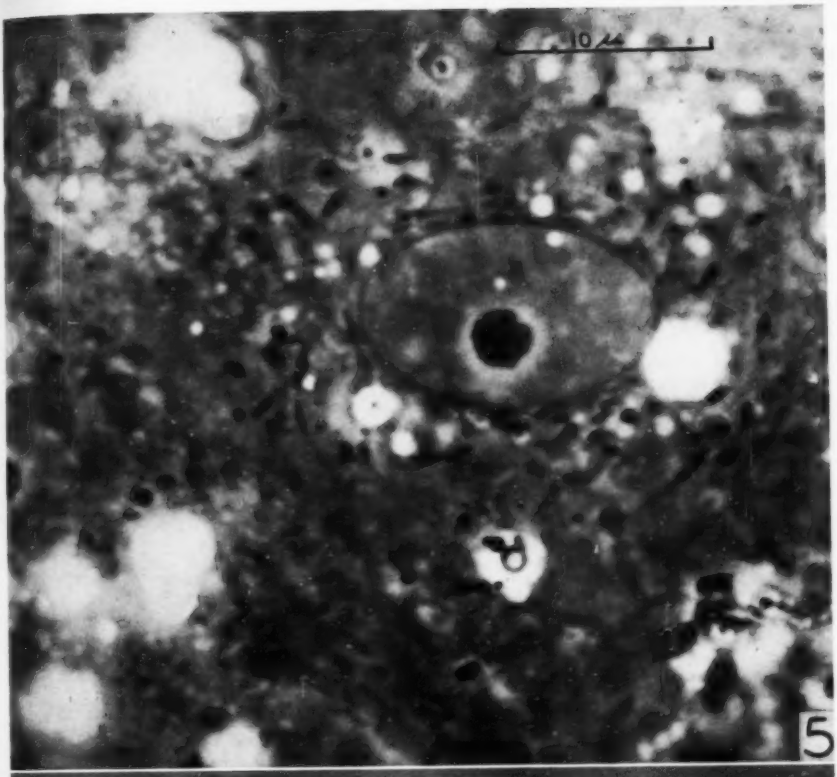
FIG. 5. Phase contrast photomicrograph of a living cell from an epithelial sheet growing out of an explant of an 11 day chorio-allantoic membrane. The culture was set up on a coverslip over a Maximow slide and incubated for two days. The coverslip was then placed over a drilled slide to enable the photomicrographs to be made. Central oval nucleus, lobulated nucleolus. Mitochondria elongated. Refractile fat droplets appear larger than in reality. Nucleus appears larger than would be expected because the cell has flattened out in tissue culture. Magnification: 3,200×

FIG. 6. Phase contrast photomicrograph of an unstained one micron section of a 12 day chorio-allantoic membrane fixed with osmic acid. Magnification: 2,100×.

c. Chorionic layer consisting of a capillary plexus lying outside of a layer of epithelial cells.

m. Mesodermal layer. Typical small blood vessel in center surrounded by loose areolar connective tissue.

a. Allantoic layer lining the allantoic cavity.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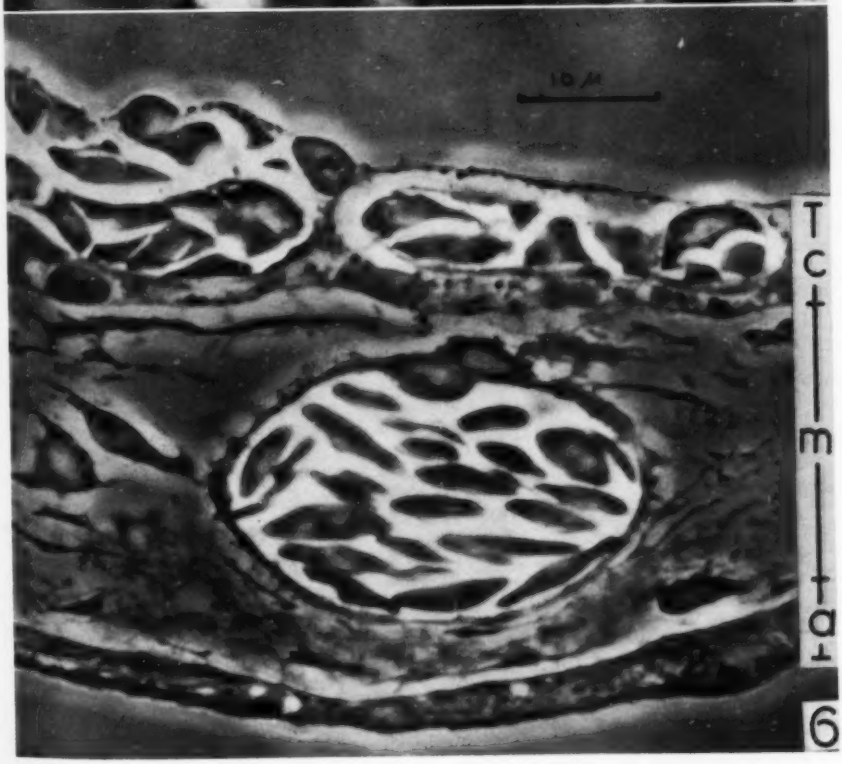
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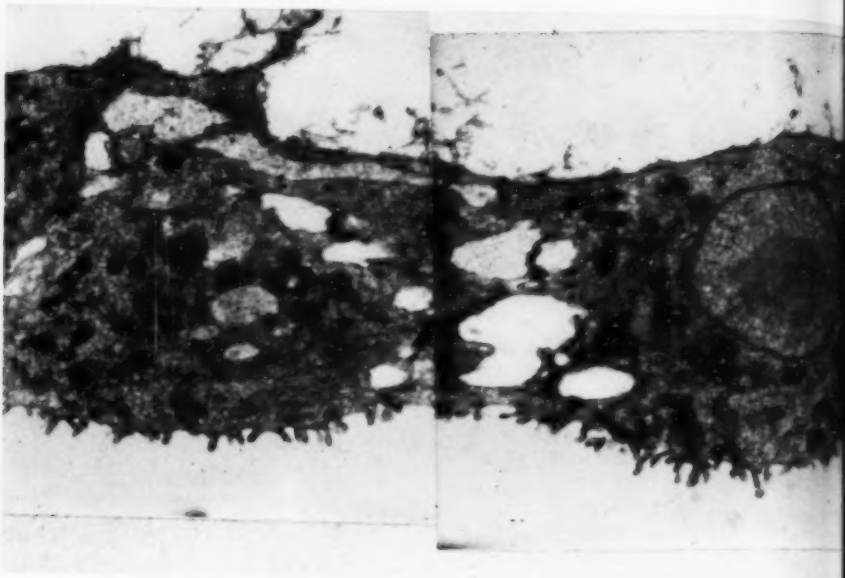

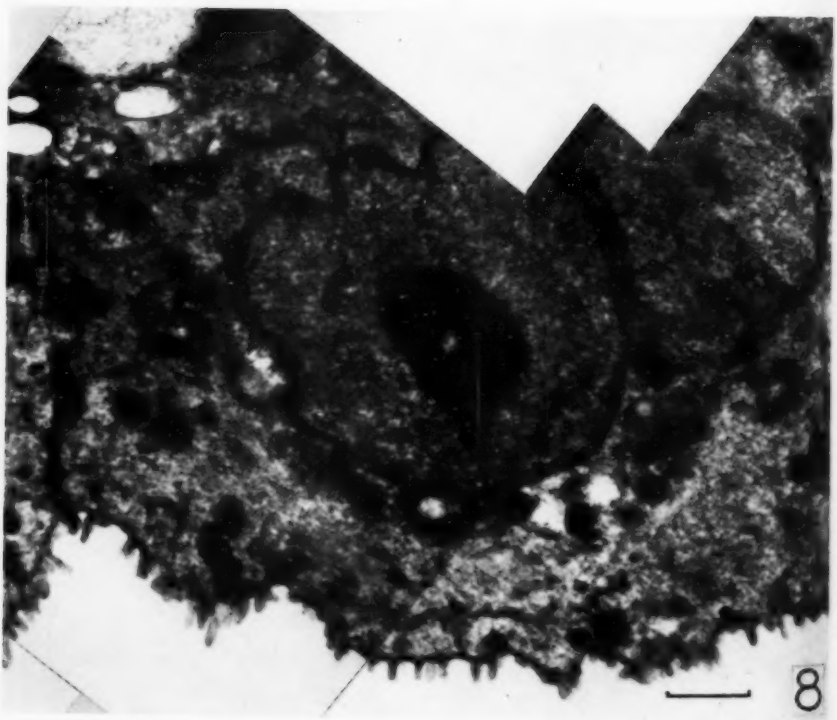
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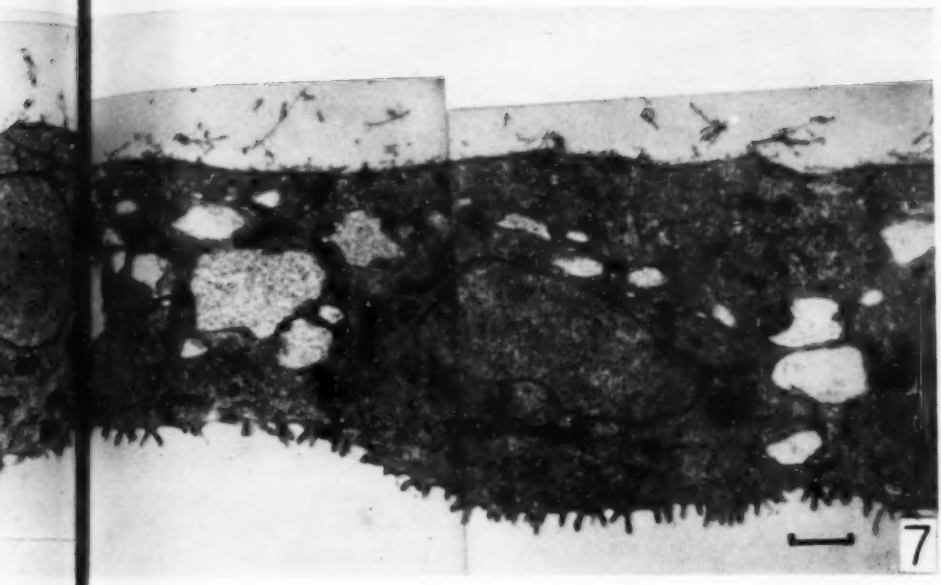

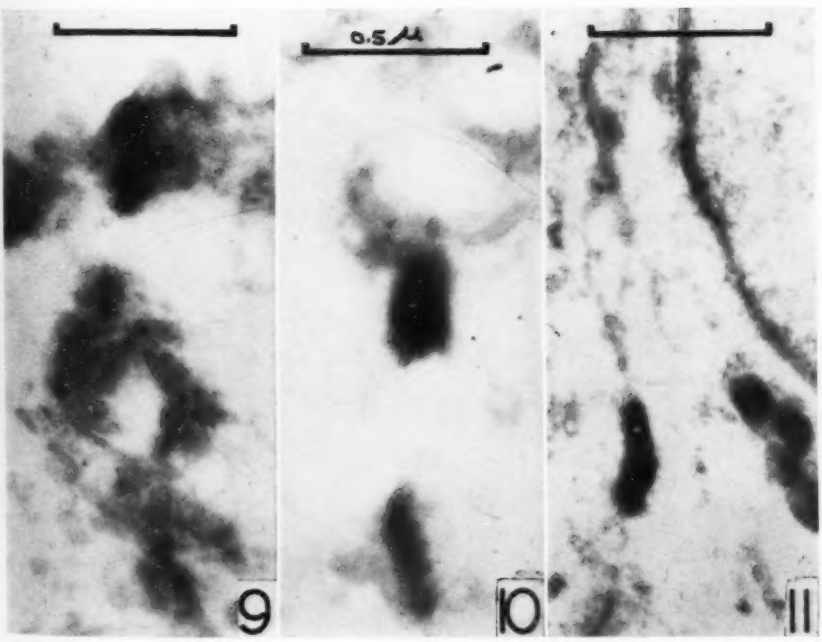

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PLATE III

FIG. 7. Cells of the allantoic layer, fixed in 2% osmic acid. Microvilli projecting into the allantoic cavity. Large vacuoles at boundaries between cells. Several dense intercellular granules can be seen. Structure of mitochondria distinct. Fat droplets, nuclear and plasma membranes well preserved. Magnification: 9,760×.

FIG. 8. Cell of the allantoic layer. Deeply indented nucleus. Cell boundary at left delineated by dense granules, one of which protrudes into the allantoic sac, and vague labyrinthine areas. Structure of the mitochondria obscured by the density of the print. Magnification: 11,000×.

FIGS. 9–11. Underexposed prints of dense intercellular granules between cells of the allantoic layer. Fig. 9 shows structure of a granule at the free surface of the cells and a labyrinthine area which is commonly found between the cells. Magnification: Figs. 9 and 11, 24,000×; Fig. 10, 44,000×.

PLATE IV

Microvilli found on cells lining the allantoic sac.

FIG. 12. Slightly greater variation in length of microvilli than is seen in the average preparation. Magnification: 12,000×.

FIG. 13. Surface of a flattened epithelial cell from roller tube tissue culture preparation. Portion of cell in left part of figure is covered with microvilli while neighboring cell at right has none. Shadowed with chromium after osmic acid fixation and harvesting from tissue culture. Magnification: 12,000×

FIG. 14. Dense cell showing numerous irregular and stubby microvilli. Cells of this type, in which little or no structure can be seen, were frequently observed in the allantoic layer. The number of microvilli on their free surfaces was extremely variable, ranging from one or two to the complete coverage shown here. Magnification: 12,000×.

FIG. 15. Unusual clump of microvilli lying over a dense, elongated cytoplasmic body. Magnification: 12,000×.

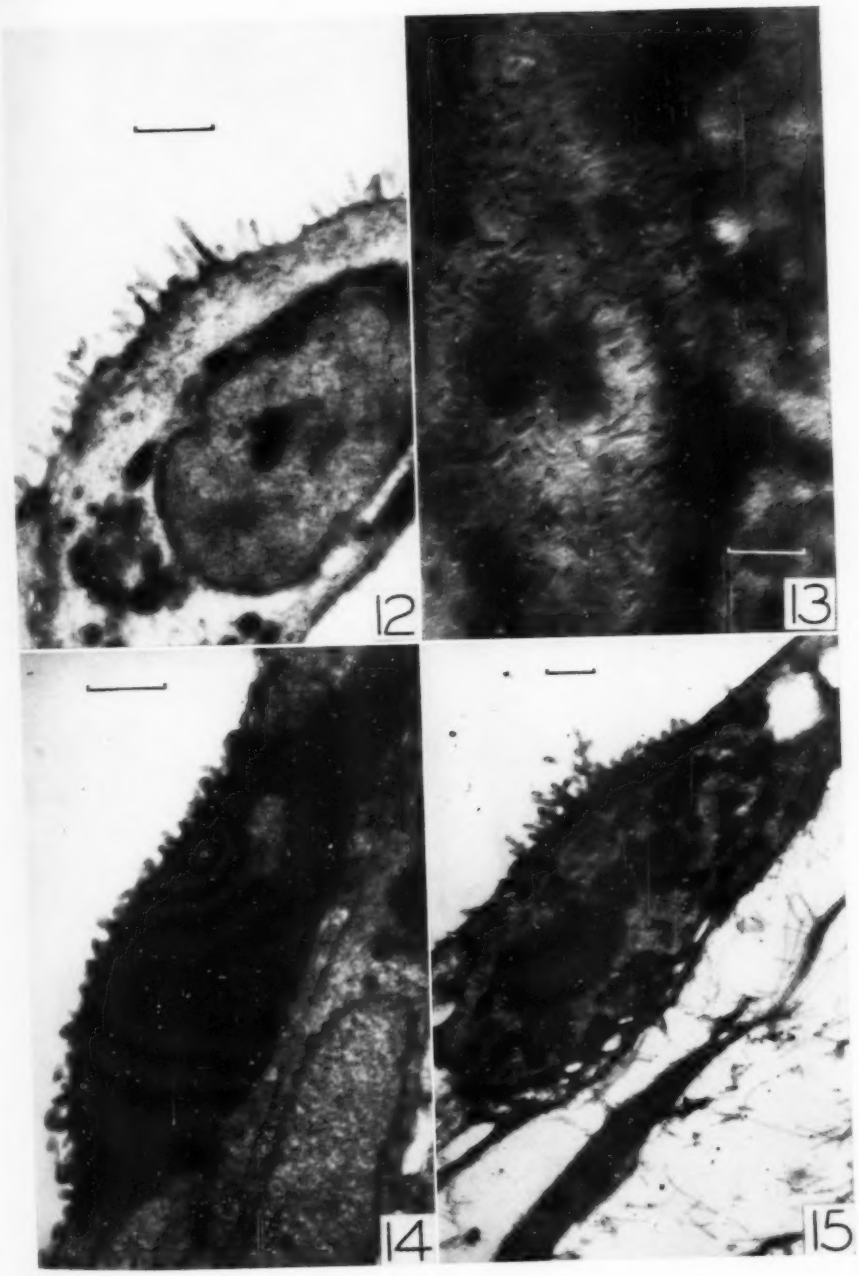
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PLATE V

FIG. 16. Enlargement of area from Fig. 7. Branching tubular "reticulum" in upper portion of picture. Mitochondria show external membrane and internal structure. Nuclear membrane appears double in some areas. Intercellular vacuoles and granules in lower portion of picture. Plasma membrane bordering on the mesodermal layer (bottom of picture) appears to be double. Magnification: 30,000×.

16

PLATE VI

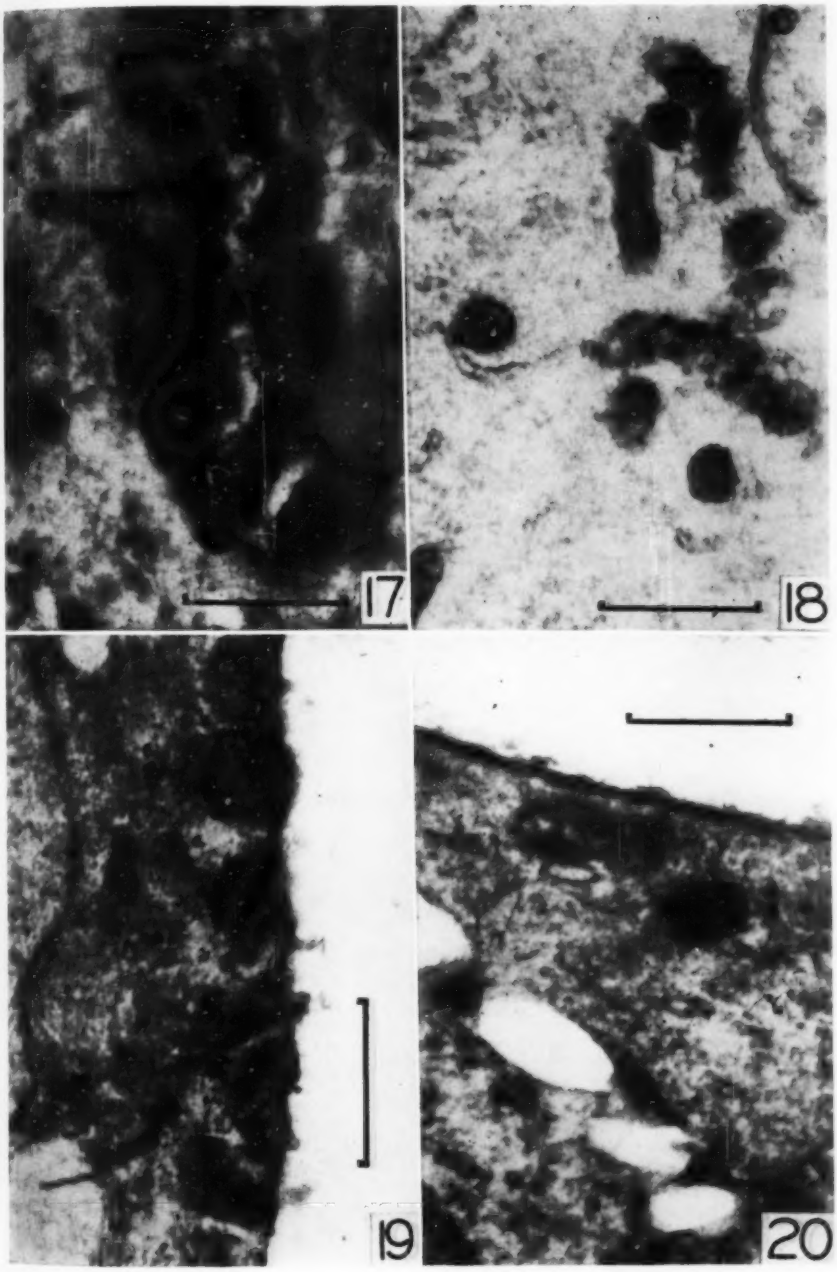
Varieties of large cytoplasmic structures in the allantoic layer.

FIG. 17. Agranular fat droplets and mitochondria showing a suggestion of structure. Magnification: 24,000×.

FIG. 18. Mitochondria with granular structure. Longitudinal section and several cross sections of tubular endoplasmic reticulum. Nuclear membrane at upper right. Magnification: 24,000×.

FIG. 19. Accumulation of granular bodies at free surface of a cell. Magnification: 24,000×.

FIG. 20. Mitochondria with typical membranous internal structure. Note doubleness of plasma membrane in upper portion of picture. Intercellular vacuoles and granules in lower part of picture. Magnification: 24,000×.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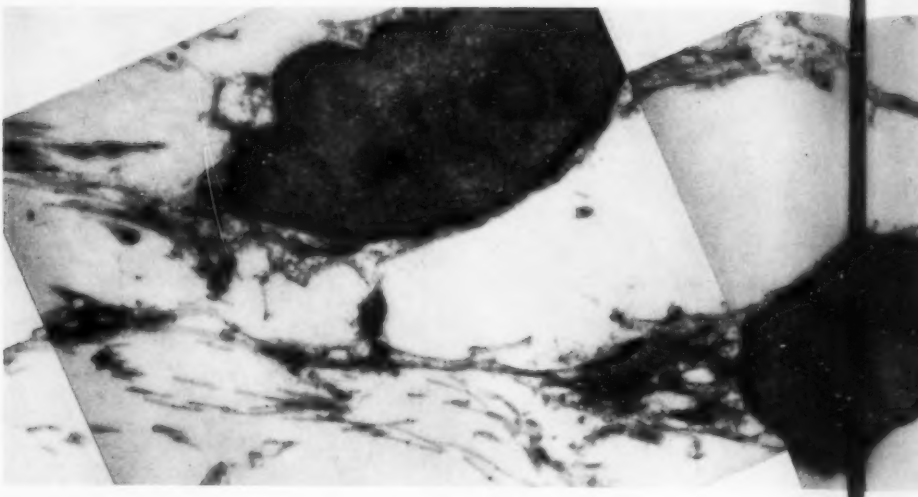

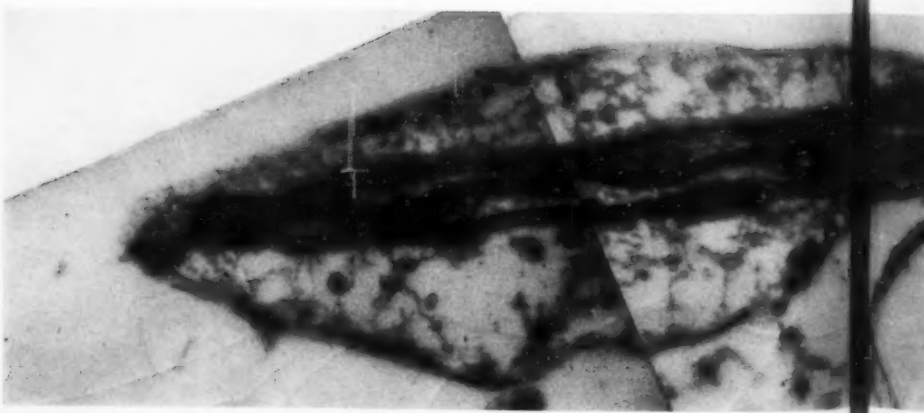

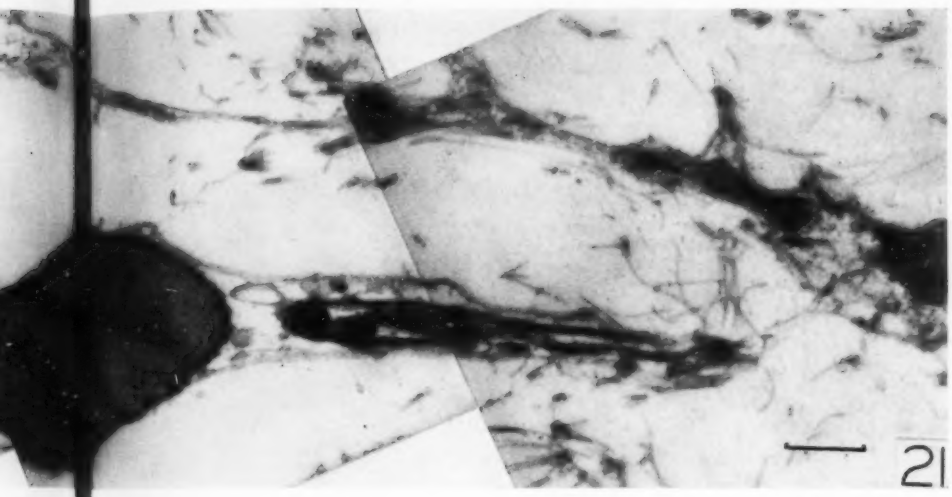

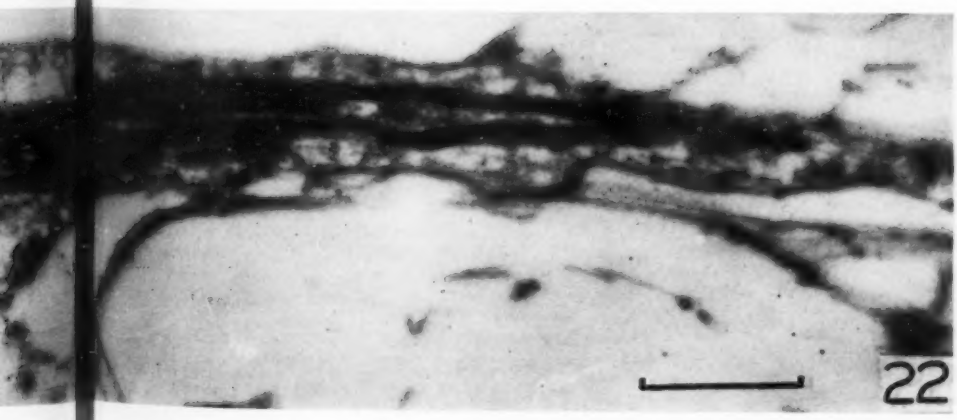

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PLATE VII

Fibroblasts in the mesodermal layer.

FIG. 21. Illustrating the stellate shape of the fibroblasts. Note the extreme thinness of the cytoplasmic extensions and the lack of orientation of the collagen fibers with respect to the cell surfaces. In the cytoplasm, long intracellular ribbons can be seen looping around mitochondria. Nuclei essentially the same as those in the allantoic layer. Magnification: 12,000×.

FIG. 22. Intracellular ribbons in a fibroblast. The extreme length in the plane of the section makes it highly unlikely that they are tubular in cross section. Magnification: 24,000×.

PLATE VIII

FIG. 23. Small blood vessel in the mesodermal layer. Typical nucleated erythrocyte in the lumen. Endothelial cells bulging into the lumen. Blood vessels of the same diameter were seen whose walls consisted of a thin sheet of cytoplasm from a single cell. Magnification: 7,700×.

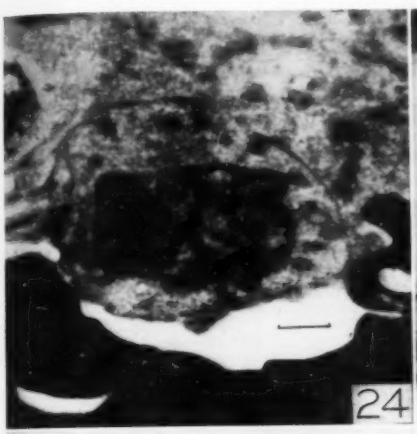
23

PLATE IX

FIGS. 24 and 25. Endothelial cells lining the blood vessels which are probably changing into red blood cells. The dense masses are mature red blood cells in the lumen of the vessels. Magnification: 7,400×.

FIGS. 26 and 27. Immature red blood cells in the blood vessels. Note the similarity of their nuclei to that of the endothelial cell in Fig. 25. Magnifications: Fig. 26, 7,400×; Fig. 27, 3,100×.

FIG. 28. Swelling of red blood cell caused by formalin fixation followed by methacrylate embedding. Though the cytoplasm has swollen to many times its original volume, the size of the nucleus has remained unchanged. Magnification: 8,100×.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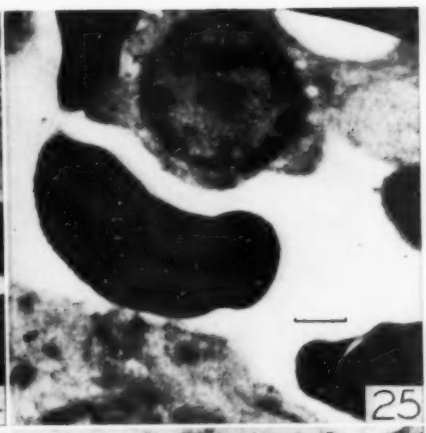
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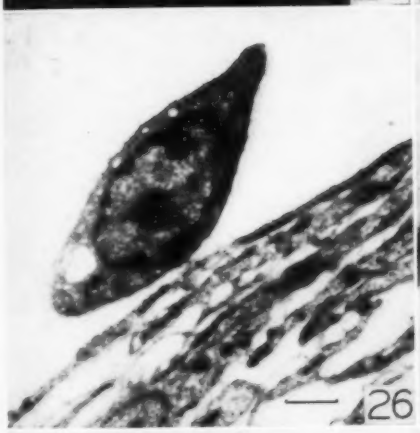
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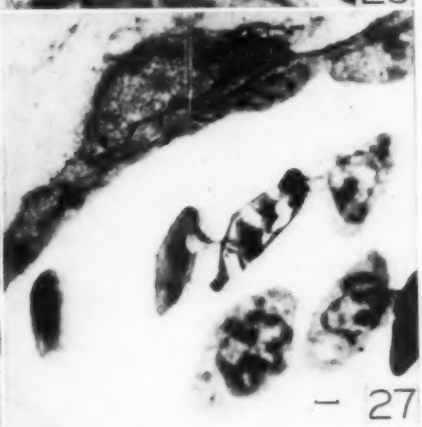
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PLATE X

Fig. 29. Chorionic surface showing flat epithelial cells lying beneath the capillary plexus. Note the presence of typical mitochondria in the extremely thin capillary wall at the left. Magnification: 5,100×.

FIG. 30. Cuboidal epithelial cells in the chorionic layer. Capillary plexus lacking in this region. Two cells have apparently ingested red blood cells. Magnification: 5,100×.

FIG. 31. Capillaries of the chorionic surface. No epithelial cells are apparent in this area. Magnification: 5,100×.

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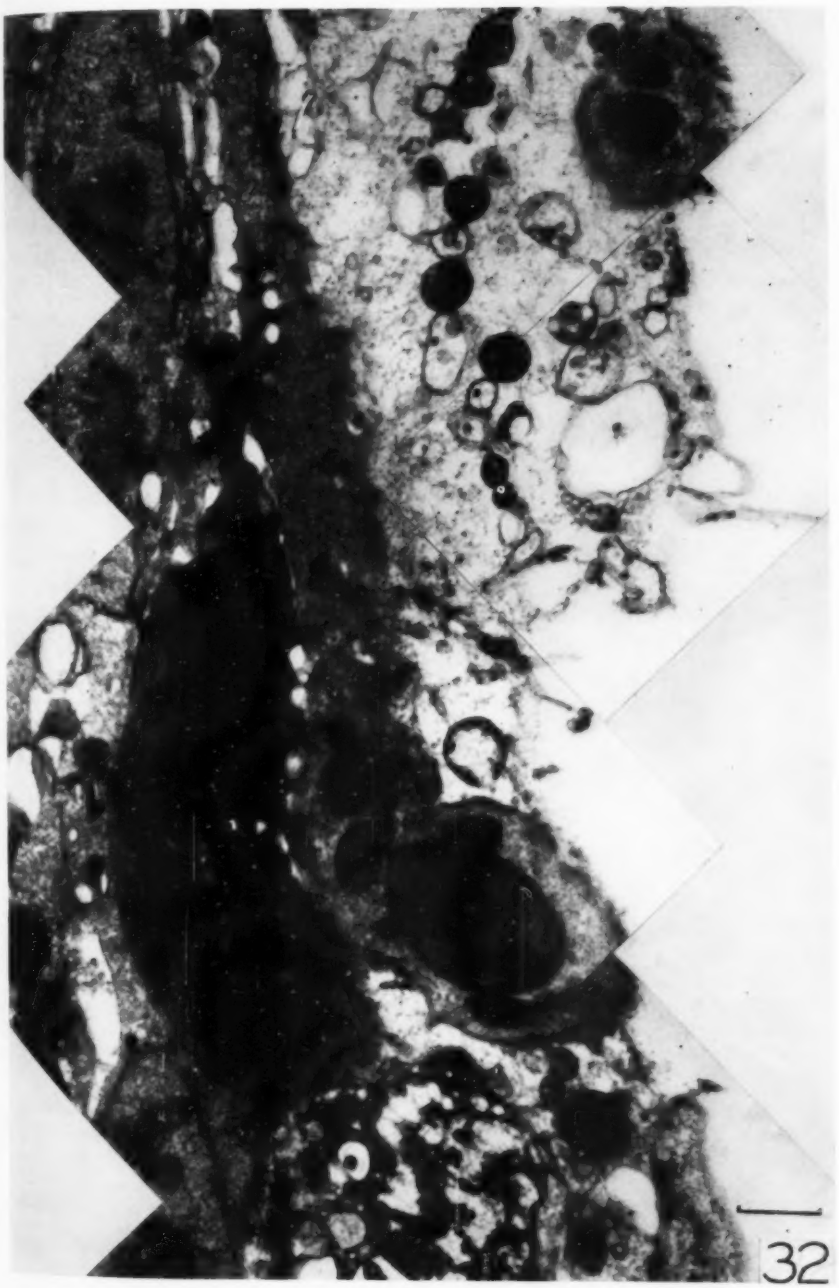
PLATE XI

FIG. 32. Cell damage in the chorionic layer caused by dropping the chorio-allantoic membrane away from the shell membrane before fixation. Note the alveolar appearance of the damaged cells. Magnification: 12,000×.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THE DEVELOPMENT OF THE VIRUS OF NEWCASTLE DISEASE IN EPITHELIAL AND FIBROBLAST CELLS IN TISSUE CULTURE

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Electron microscope study of cells in tissue culture (1) or in thin sections (2, 3) may eventually contribute to our knowledge of the *development* of virus within or on specific host cells. Studies on the development of Newcastle disease virus in chick embryo tissue cultures (4), and in thin sections of chick chorio-allantoic membrane are presented here. A few pictures of filaments projecting from cells have been previously published (5) and Kilham and Wyckoff (6) have pointed out filaments extending from cells into the allantoic fluid in sections of infected membranes.

The development of the virus in tissue culture has been studied by three methods (Chart I) which have together led to the following conclusions: a) Epithelial cells may form long delicate projections from the surface of the individual cells. The projections apparently contain formed and partially formed virus particles. b) These characteristic "virus filaments" are present before morphological changes are detected in the substance of the cell. c) Their formation may be an exaggeration of the normal process by which the cell forms the short stubby surface projections, which we have called microvilli. d) Fibroblasts on the other hand are usually destroyed with a release of scattered virus particles. Filaments may be formed, but less frequently.

METHODS

Three standard strains of Newcastle virus have been used. They are the virulent strain (CG179) which is the most rapidly destructive for embryo tissues (7). Its counterpart "B" does not kill adult chickens (8) or mice (9) but does rapidly invade the chick embryo. The third strain the "Blacksburg" or Hitchner strain (10) will be referred to as the "vaccine" strain since it is the least virulent and is currently used as a live vaccine for newly hatched chicks.

Three general methods of preparation of thin cells in tissue culture for electron microscopy are shown in the diagrams of Chart I. They are standard techniques and were used in a previous study of the development of the virus of influenza (11). However, since the techniques of culture have not remained exactly as before, each method is briefly presented in the particular section.

Unless otherwise indicated 25 per cent serum from chickens known to be free of Newcastle infection was used. Sera from the same source were constantly used in the laboratory

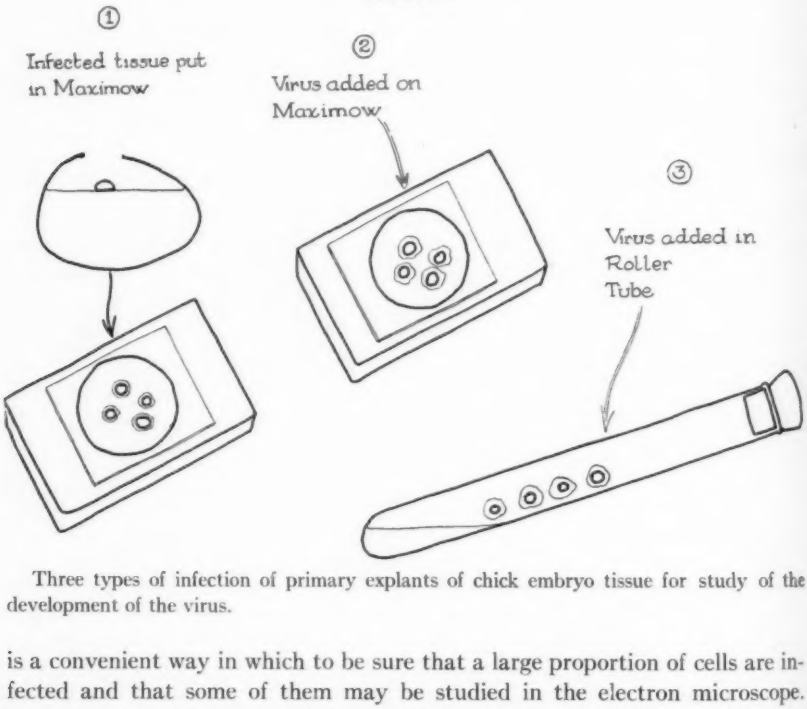
Supported by a Grant-in-Aid from the National Institutes of Health, U. S. P. H. S., Bethesda, Maryland.

for other tests and were found to be free of antibodies against Newcastle virus. No penicillin or streptomycin was added to the cultures. All three tissue culture methods were used in the study of the "B" strain, but all were not used in studying the other two strains. Most of the electron microscope preparations were unshadowed, but on occasion they were lightly coated with chromium (12) to increase the contrast.

Coverslip Preparations

Infection before in vitro cultivation—This is really a preparation of epithelial cells from infected embryos. Bits of chorio-allantoic membrane are explanted onto prepared coverslips so that the cells will migrate out in a thin sheet. This

CHART I



Three types of infection of primary explants of chick embryo tissue for study of the development of the virus.

is a convenient way in which to be sure that a large proportion of cells are infected and that some of them may be studied in the electron microscope. However, it is not known whether cells so explanted from infected embryos continue to yield new virus or whether the migrating cells merely carry with them the virus which has already been formed in the chick embryo.

METHODS

Six satisfactory experiments were performed. Ten day old chick embryos were infected by placing one drop of a 10^{-3} dilution of Newcastle virus (approximately $10^{5}LD'_{50.5}$) on the chorio-allantoic membrane after the membrane had been lowered so that its upper surface

might be infected through an artificial window in the shell. Following inoculation, the epithelial cells lining the allantoic sac (the lower surface) also frequently became infected. The chorio-allantoic membrane was removed from the embryo after 24 hours' incubation at 35°C. and bits of tissue were placed on formvar covered slides. In two of the experiments (Fig. 1) the tissues were grown in drilled slide preparations. These have relatively little air space, since the fluid touches both sides of the enclosed sandwich preparation. In the other four experiments, tissue was placed directly on formvar-coated coverslips which were subsequently inverted over Maximow slides. These primary explants produced a mixture of epithelium, fibroblasts and macrophages, all in varying stages of destruction.

RESULTS

Before considering the results of the tissue cultures, the morphology of the virus itself should be reviewed. Preparations of the virus dried from saline and washed have been found to contain sperm-shaped or tadpole-shaped forms of the virus (13–16). These have not been found in the allantoic fluid or in aqueous solutions dried for electron microscopy. The form of the virus changes when it is placed in hypertonic saline or when dried from normal saline. The change may be prevented by fixing the preparation with osmic acid before it is dried (17). A large variation in shape, then, is produced by variations in salt concentration. Since many of the elongated forms of the virus prepared in saline were thread-like in form, they were called filamentous. The diameter of these forms is usually much less than that of the spheres and the two extreme forms seem to be interchangeable in varying salt concentrations.

Simultaneous with the description of these forms of Newcastle virus (13) Mosely and Wyckoff (18) described certain long thread-like forms of influenza which occur in the allantoic fluid. These forms have a *uniform diameter which is the same as the spherical forms of virus*. They are now well known as the filaments of influenza virus. Our present studies indicate that Newcastle disease virus will give rise to long threads extending from the cell's surface, and that spherical forms may arise within these cell extensions. These threads may break off and be found in the allantoic fluid. They should then also be called the filaments of Newcastle disease virus. It therefore seems advisable to abandon the term "filaments" or "filamentous forms" as applying to the pleomorphic variation in shape which occurs in saline with washed preparations of this virus, and restrict its use to the larger and often longer forms which, like influenza, occur in the allantoic fluid. We may then clearly differentiate between varieties in shape induced by saline and varieties which represent developmental forms. This distinction has not always been clear (19). The pleomorphic nature of the washed purified virus may well have a significant relation to the development of the virus in the cell, but the relationship of this *in vitro* phenomenon to the filaments of the allantoic fluid is not known.

The knowledge that the virus has a spherical form in physiological saline and in allantoic fluid then led us to look for spherical forms of virus in the tis-

sue cultures. Such forms, about 100 to 120 mμ in diameter, were found scattered throughout the infected cultures. However, a variety of rods and long threads having a similar diameter, but with bulbous endings, were also found (Figs. 1 and 2). The rods seemed in some fields to contain material of varying density, with sharp breaks in the continuity of the denser portions. The ends of the filaments were frequently bizarre in form; in some the denser portion of the filament seemed to continue into a sac-like balloon (Fig. 3), in others the enlarged whole tip was dense and was in contact with groups of the usual spherical form of the virus. Finally some of the filaments consisted of a series of roughly spherical forms connected together rather loosely as a chain (Fig. 4).

Infection of Coverslip (Maximow) Preparations after Growth in Vitro

It was not possible in the above experiments to compare the amount of development of the virus which occurred in the cultures with that which occurred before the infected tissue was transplanted. Therefore, epithelial cells from the chorio-allantoic membrane were grown on formvar-coated coverslips for two days and then infected.

METHOD

One ten day old chick embryo furnished sufficient tissue for all of the slides in one experiment. The chorio-allantoic membrane was removed into sterile saline and cut into small pieces. About four pieces were placed, in 25 per cent chicken serum, on each of eight or ten formvar-coated coverslips. The Maximow slide was then placed over the preparation and was sealed to the coverslip by a small drop of sterile saline at each corner. The use of vaseline was avoided since it may spread over the culture at the time of harvesting and give rise to scattered small "grease balls". After two days' incubation at 37°C. those cultures which showed good growth of epithelium were selected for the experiment. Each experiment included two cultures inoculated with undiluted infected allantoic fluid, and two with a 10^{-3} dilution of this fluid. There also were two uninfected control cultures. Following virus inoculation, the cultures were incubated again for 24 or 48 hours at 37°C. They were washed briefly in normal saline at 37°C. and fixed for 15 minutes with vapor from two drops of a 2 per cent osmium solution placed in the Maximow slide. After this they were washed first in saline and then in water. It was necessary to harvest them within an hour after this washing to prevent cellular disintegration in the water.

Five satisfactory experiments were performed. The epithelial cells which were grown in such cultures had a smooth surface, and lacked the microvilli which were common on the same cells grown in roller tubes. Two types of structures were seen in the infected preparations which were believed to be part of the host cell-virus complex. A series of small collapsed sacs with dense areas within them were found plastered on the surface of individual epithelial cells. They were often found in large numbers on individual cells, while neighboring cells had few or none. The sacs were identical in size, shape and appearance with the virus found free in the allantoic fluid or adsorbed on to red cell ghosts.

The long thin filaments seen as remnants of the destroyed cells in the pre-

infected cultures were also found. They were particularly prevalent and long in a culture infected with the "vaccine" strain of virus (Fig. 7). Whether this strain characteristically produced this effect was not determined. These filaments were usually very uniform in width, but were ribbon-like in contrast to the uniform spaghetti-like forms of influenza virus. In some preparations these ribbons appeared to be double through their entire length, and in others appeared twisted or doubled upon themselves. Despite this morphological evidence of virus effect, it has been difficult to demonstrate an overall increase in the amount of infectious units in the fluid from cultures of this type (Table I). This may be because a large portion of the virus is bound to the tissue. Furthermore, the titrations measure the amount of discrete virus units and not the mass of the virus protein. Thus a spherical virus particle would have the same effect as an elongated ribbon of ten to twenty times greater length.

TABLE I

Amount of Virus Recovered from 11 Day Old Chick Chorio-Allantoic Membrane Explants Grown in 25 Percent Chicken Serum

(Maximow slides incubated at 35°C.)

EXP.	INOC.	HOURS AFTER INOCULATION						
		4	6	8	12	18	24	30
1	10^{-6}		1.6			<2.5	2.0	<2.5
2	10^{-5}	2.8		2.5	2.3		<1.5	
		<1.5		<1.5	2.5		<1.5	

Roller Tube Preparations

All three strains of NDV here studied multiply readily in roller tube cultures of both epithelium and fibroblasts (Charts II, III). For this reason we were particularly interested in studying the development of the virus in such cultures.

METHOD

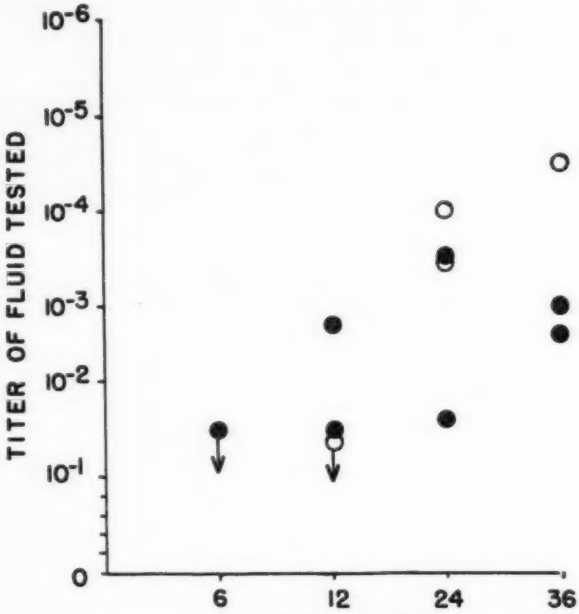
Again the method of preparing epithelial cultures of the chorio-allantoic membrane was similar to those used in the study on influenza virus. The bits of chorio-allantoic membrane were placed on formvar coated roller tubes, and were allowed to grow for one to two days before being inoculated with varying dilutions of virus. Cells were fixed with vapor formed from one or two drops of 2 per cent osmium solution which was allowed to run down the side of the roller tube opposite the cultures. The harvesting procedure was the same as that previously described (11). In two cases epithelium was obtained from 10 day old embryo skin. Cultures consisting predominantly of fibroblasts were obtained from leg muscle.

RESULTS

As in the influenza study (11) uninfected epithelial control cultures studied in the roller tube showed many projections of the cell surface, often great

masses of them. Although these microvilli (20) prevented observation of the early stages of virus development, they are short and could be distinguished from the long virus filaments which developed after some hours. Some infected cells showed a mixture of the two with spherical virus appearing as a chain

CHART II



COMPARISON OF THE GROWTH OF N.C. VACCINE 10^{-4} VS. N.C. CG 10^{-4} ON PRIMARY EXPLANTS OF EPITHELIUM FROM CA MEMBRANE OF 10 DAY CHICK EMBRYO IN ROLLER TUBES

● CG ON EPITHELIUM

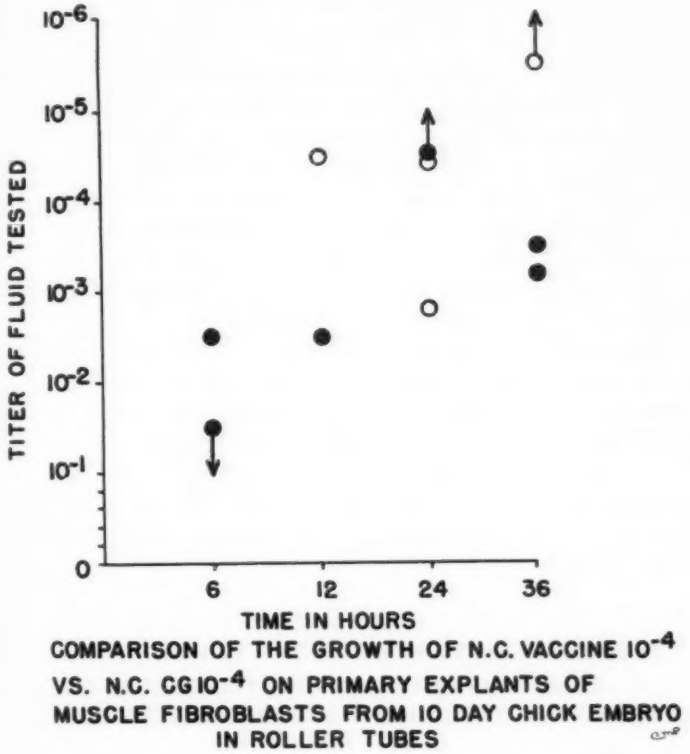
○ VACCINE ON EPITHELIUM

within the filaments (Fig. 9). Fibroblast cultures were studied more intensively since normal fibroblasts in roller tube cultures failed to show such surface projections.

Eight experiments with roller tube cultures of fibroblasts were completed. Although it was possible in all of these to induce great destruction of the cells

and to find masses of apparent virus in destroyed cells or in the fibrous skeleton of the cell (Fig. 8), we could not follow the development of the infection when the usual inoculum of virus was used. At this writing, several preliminary experiments with large amounts of virus have been encouraging. One drop of a concentrate containing about $10^{11}LD_{50}$ was put into two day old roller tube

CHART III



preparations of fibroblasts. The virulent strain (CG179) was used exclusively in these experiments. Cells were harvested at short intervals thereafter. With this larger inoculum large amounts of virus could be found immediately after inoculation, often distributed in a surprisingly even fashion over both cells and background membrane (Fig. 11). Although some cells survived such doses

of virus for as long as 24 hours, many showed ragged areas of degeneration and destruction within six hours after inoculation (Fig. 12). Others showed characteristic virus filaments extending both from the surface and the edge of the cell (Fig 10).

Such "virus filaments" also have been seen in a roller tube culture of fibroblasts inoculated with a standard dose of virus and in a closed sandwich type of slide of pre-infected fibroblasts. They are, however, unusual on fibroblasts.

DISCUSSION

An attempted interpretation of the development of *virus infection* will be presented in the following paper. We will, however, discuss the tissue culture methods here. The thin spread of the cytoplasmic portion of the cell which occurs in tissue culture offers unique opportunity for study with the electron microscope (1). There have been relatively few viruses studied with the combined techniques of tissue culture and electron microscopy (5, 11, 21, 22, 25). Notable pictures of tumor cells with apparent virus (23, 24) have been published, but since the tumors contained the virus before they were cultured, studies on cellular pathogenesis were not feasible.

The identification of a virus in tissue culture is not easy. The size, shape, density, and arrangement of the particles contribute, in concert, to their identification. It is not enough to examine normal uninfected cells. Controls include the same cell types but infected with other viruses.

It has been pointed out (19) that degenerating red cells may form filaments of several kinds and shapes. It has also been indicated that virus "filaments" are artifacts of red cell destruction. We have obtained no evidence associating these *virus induced filaments* with red cells.

SUMMARY

A study of chick embryo cells grown on formvar-coated glass slides or roller tubes, and infected with different strains of Newcastle disease virus, has led to the following conclusions: a) Infected epithelial cells may form long delicate projections from the surface of the individual cell. The projections apparently contain formed and partially formed virus particles. b) These characteristic "virus filaments" occur before morphological changes are detected in the substance of the cell. c) Their formation may be an exaggeration of the normal tendency of the cell to form short stubby surface projections. d) Fibroblasts on the other hand are usually destroyed, releasing scattered spherical virus particles. They form filaments, but less frequently.

ACKNOWLEDGEMENTS

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PLATE I

Newcastle disease virus in explants of infected chick chorio-allantoic membrane. Osmic acid fixation. ×12,000.

FIG. 1. Scattered spherical and short filamentous forms. Drilled slide preparation.

FIG. 2. Predominance of filamentous forms. Note interrupted appearance of dense material which seems to be contained within an envelope.

FIG. 3. Large, dense spherical masses at tip of filaments. These balloons may be compared to Figure 15 in the accompanying paper on sectioned material.

FIG. 4. Long irregularly segmented and pinched filaments with roughly spherical forms within filaments. See Figure 24 in accompanying paper.

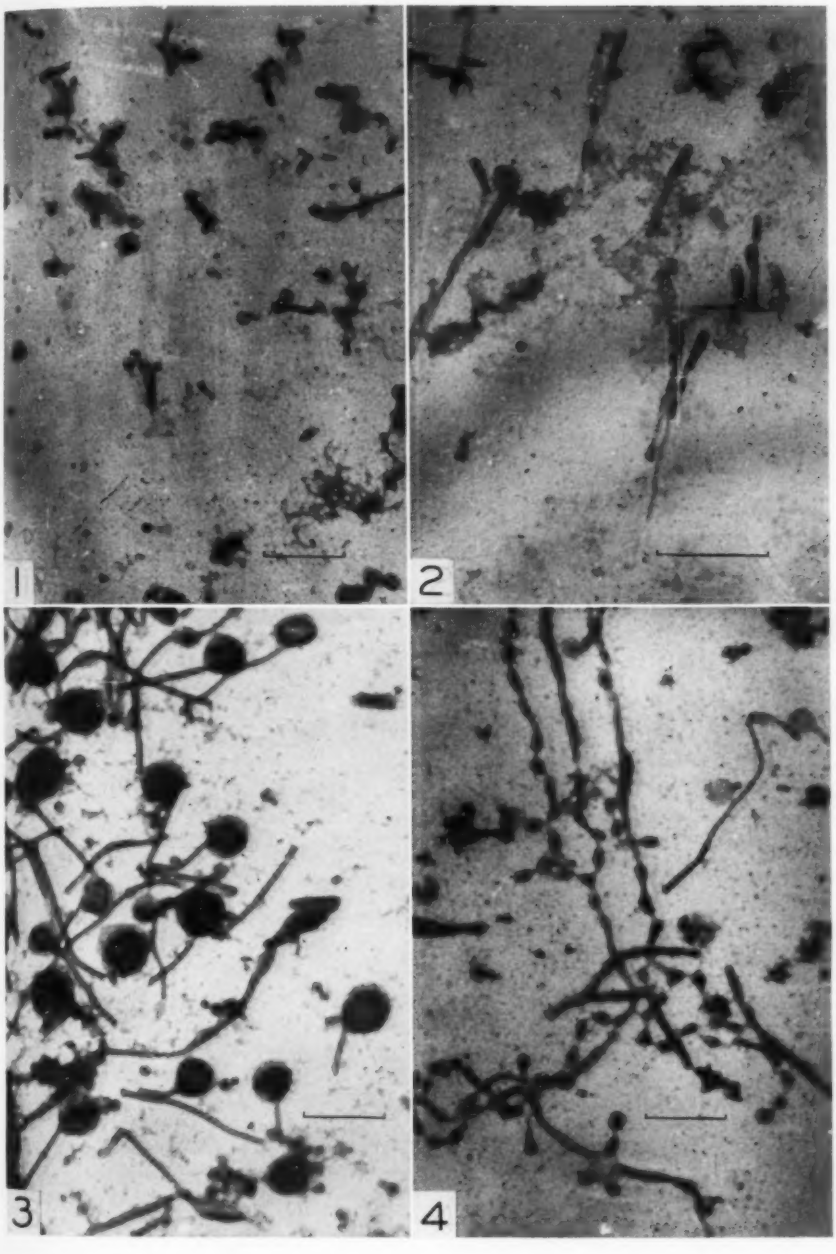
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PLATE II

Infection of epithelial cells in primary explant of chick chorio-allantoic membrane. ×12,000.

Procedure—Cells were explanted on Maximow slides in 25 per cent serum, grown in this static medium for two days and then infected with virus. They were harvested 24 to 48 hours thereafter.

Fig. 5. Scattered spherical particles varying between 100 to 200 mμ on surface of epithelial cell at edge of explant. B strain. Particles below this size cannot be differentiated from normal cell particles.

Fig. 6. Infected epithelial cell. Large hole in center is artifact in supporting screen with break through of cell. Dense ridge running through center of figure marks edge of infected cell. Cells to left have relatively little infection. B strain.

Fig. 7. Long filamentous forms on surface of cell. These are longer, thinner, denser and more regular than the normal microvilli. Vaccine strain.

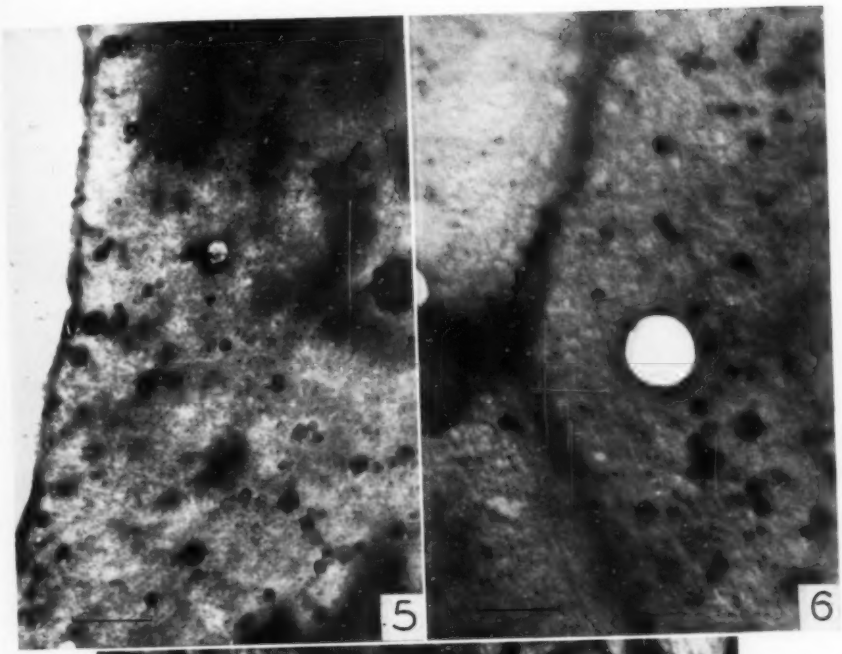
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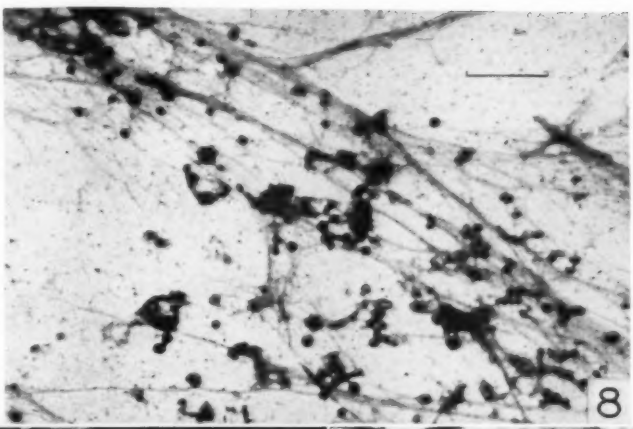
PLATE III

Newcastle disease virus in roller tube cultures.

FIG. 8. Destroyed fibroblast cell after 24 hours growth of virus. Only skeleton of cell and scattered dense bodies, presumably virus, remain. ×12,000.

FIG. 9. Epithelial cell with short chains of virus attached to altered thickened microvilli. ×12,000.

FIG. 10. Filaments occurring on fibroblasts following heavy infection lasting 24 hours. ×9,700.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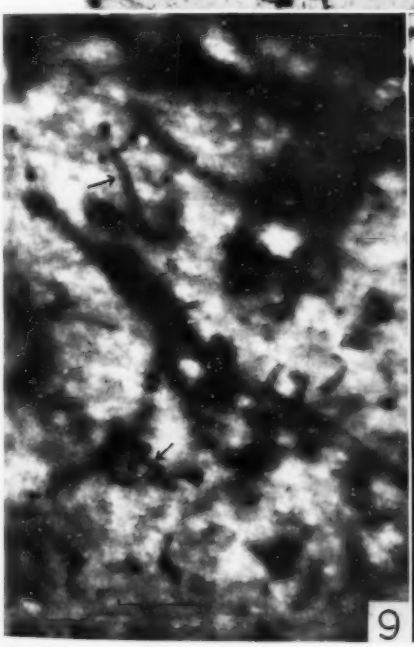
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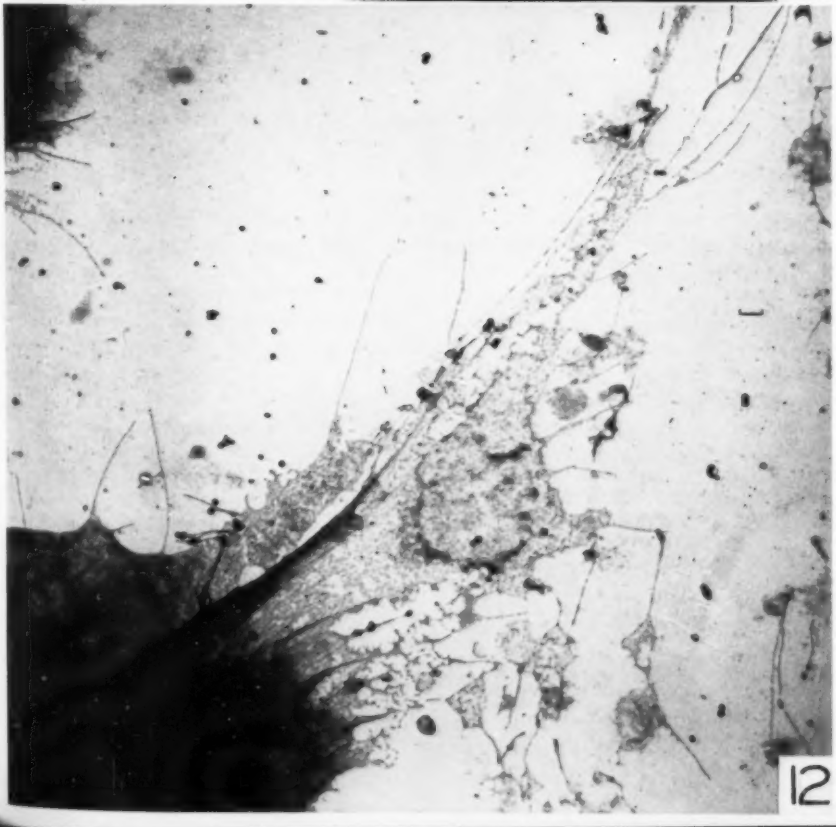
PLATE IV

Effect of large doses of virus on fibroblast cells in roller tube cultures.

FIG. 11. Cells harvested one-half hour after addition of virus. ×3,400

FIG. 12. From same experiment. Cells harvested 6 ½ hours after addition of virus. ×3,400

11



12

THE DEVELOPMENT OF NEWCASTLE DISEASE VIRUS IN CELLS OF THE CHORIO-ALLANTOIC MEMBRANE AS STUDIED BY THIN SECTIONS

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The microvilli so characteristic of normal allantoic epithelial cells of the chick chorio-allantoic membrane (1) may well have an important role during a virus infection. This paper will describe their reaction to infection with Newcastle disease virus, and will discuss the reaction as a part of the whole complex of host-parasite relationships. This paper presents evidence that Newcastle disease virus causes the normal microvilli to enlarge and extend, and that the virus then exits from the cell by means of these modified microvilli*. This may be demonstrated by electron microscope study of thin sections of infected membrane. Different strains of Newcastle virus have greatly different effects on cells. The virulent (CG) strain seems to develop deep in the cell and to destroy it rapidly. Heavy inocula of this strain apparently lyse the microvilli soon after infection. In contrast, the "vaccine" strain induces massive epithelial hyperplasia and little cell destruction. This hyperactivity appears the second day of infection and may continue for five to six days.

METHODS

Methods of fixation and treatment of infected membranes were identical with those used in the study of normal membranes (1). Sections of uninfected membranes were prepared simultaneously with those of infected membranes so that a given batch of methacrylate, a particular procedure of embedding, etc., was controlled by a parallel preparation of a normal membrane. All sections were cut by Mr. Emil Borysko. An average of ten satisfactory sections from each experimental infection was examined with the electron microscope.

The same three strains of virus used in the tissue culture study (4) were used, and the standard infection was produced by inoculating virus into the allantoic sac of 11 day old embryos. Undiluted or 10^{-3} dilutions of allantoic fluid were used routinely. The embryos thus received from 10^5 to $10^8 LD_{50}$ of virus. With either dosage, the three strains all reached a high titer in the allantoic sac within 36 hours (5). Therefore, nearly all cells lining the sac were exposed to virus after that time.

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* The "virus filaments" observed by Kilham, Morgan and Wyckoff (3) were probably these modified microvilli, but the authors were unable to follow the transition from normal microvilli to infected and modified ones.

RESULTS

Although the virus seems to infect cells deeper in the membrane and even to have a selective effect on the epithelial cells next to the shell membrane, particular attention is paid in this study to the epithelial cells lining the allantoic sac.

The Effect of the Virulent Strain—Membranes from embryos infected with 10^{-3} dilution of fluid for 24, 36 and 40 hours were examined.

This strain was rapidly destructive not only for the chick embryo itself but for the *epithelial* lining cells as well. Reaction to the virus was manifest in several ways. Large masses of dense inclusions appeared in the cytoplasm and even in the nucleus of the cell (Figs. 1, 2, 3), often without notable change in the microvilli. Many scattered dense particles appeared in other areas in the cell (Fig. 4). These measured from 60 to 120 mμ in size and were often ovoid. They were not seen in normal membranes, nor in two preparations of eastern equine encephalitis or several of influenza. Few, if any, have been found associated with the other two strains of Newcastle virus. They have been tentatively identified as virus (see discussion). With the cellular invasion there may be swelling and some destruction of mitochondria. Some areas showed swollen remnants of microvilli (Fig. 5). This observation was examined further. Large doses (0.5 cc. of 100× concentrated allantoic fluid virus—approximately 10^{12} LD_{50}) of virus were inoculated into the allantoic sac. The embryos were sacrificed after 2 and 7½ hours' incubation at 35°C. Minimal changes were apparent in the first group. However, marked changes were present in the sections from the older infection. A complete disintegration of the microvilli was associated with an apparent change in the surface membrane of the cell so that intact mitochondria seemed to be present outside the cell (Fig. 6).

As will be brought out later, the other two (less virulent) strains of virus produced long filaments extending from the cell's surface. This effect was most apparent after 48 or more hours of infection. Thus, the embryos studied with the less virulent strains tended to be older. In order to compare infection from embryos of the same age at the time of sacrifice, 13 day old embryos were infected with 10^{-3} dilution of the virulent strain. These embryos were sectioned after 36 hours of infection. The changes were similar to those in the younger embryos. Filaments were not present, and a destruction of the surface membrane with extravasation of mitochondria was apparent.

Effect of the "B" strain (chicken avirulent) on cells of the chorio-allantoic membrane—In preliminary studies of membranes infected with this strain, the usual artificial window was cut in the shell and the chorio-allantoic membrane which was dropped away from the shell membrane was then inoculated on its upper surface. Such preparations were comparable to the type of infection produced when pre-infected membranes were transferred into tissue culture

(4). In several cases cells were found to have apparent virus particles in the cytoplasm or on the surface area (Figs. 7, 8) and occasionally to have stalks projecting from the surface. However, it did not seem feasible to compare studies on the development of the virus in the allantoic fluid (5) with such infections, since separation of the chorio-allantoic membrane from its attachment to the shell membrane ruptured cells and scattered their contents over the surface of the membrane (1), thus adding to the contaminating particles in the section.

In view of this mechanical difficulty most of the studies were carried out by allantoic inoculation of the virus. Preparations were incubated at 35°C. for 36 hours and the membrane fixed *in situ*. In two such experiments the results of short fixation (15 minutes to 1 hour) and long fixation (24 hours in 2 per cent osmium tetroxide) were compared to see if osmic acid would have a digestive effect on the cell's cytoplasm and leave the virus behind. The mitochondria were well preserved. The surface membrane with its microvilli was also left intact but the general leeching out of the substance of the cytoplasm makes it difficult to attach any significance to the scattered dense particles of about 60 mμ which were left in the cytoplasm.

The most prominent effect of the virus on these cells has been mentioned and illustrated previously (6). It consisted of a profuse outgrowth of filaments (Fig. 9) containing scattered dense particles. Many of these were balooned at the tip. Sometimes these tips were swollen greatly so that the section cut through a practically empty shell (Fig. 10). Unlike the tissue cultures, it was rare to find dense material within the tip.

This strain of intermediate virulence is also destructive for the chick embryo. We previously noted that this strain may be accompanied by a filamentous outgrowth from cells without apparent cell destruction (6). This is confirmed in these studies. Many cells with filaments were however destroyed or in various stages of destruction with virus within them (Figs. 11, 13). Mitochondria within the endothelial cells lining the blood vessels of the membrane were frequently distended or destroyed (Fig. 12). If this destruction were due to growth of virus in the endothelial cells, virus could thus be released into the bloodstream.

Formalin fixed material (neutral but unbuffered formalin) showed the same general characteristics, but the outlines of the cells were hazy and no new features were apparent.

The formation of "pocks" when Newcastle disease virus is inoculated onto the exposed upper surface of the chorio-allantoic membrane is a well recognized phenomenon (7). The pocks must represent local lesions which persist for two to three days after the original inoculation. This localization may also follow inoculation into the allantoic sac, so that all the allantoic sac epithelium is not

infected simultaneously, and different parts of the membrane may have been infected for different periods of time.

Electron microscope sections through such pocks show hyperplasia, cell destruction and the penetration of leucocytes. Many of these leucocytes are disintegrating with a characteristic ballooning disintegration of the mitochondria. The mitochondria are so filled with particles that one wonders whether virus is within them. This effect on the leucocytes was also seen in the membranes infected with the CG strain of virus, and has been seen in two sections of membranes with myxoma virus. It has not been seen in the vaccine strain of Newcastle disease virus which does not produce a significant viremia (5).

Effect of the vaccine or Blacksburg strain—The third strain, the vaccine strain, of NDV may be studied for a longer period of time in the embryo. It kills the embryo only after 6–8 days of incubation (5). In the meantime it has multiplied in the allantoic sac, and attained a high titer in 36–48 hours. It may even continue to multiply in the cells lining this sac. At any rate the cells lining the sac are exposed to high concentrations of the virus from that time on. Sections of membranes from embryos infected for 24, 48*, 96, 120* and 144 hours were studied. No definite effect was seen at 24 hours. At forty-eight hours some of the microvilli were distended and swollen and a few long filaments had formed. The microvilli were not destroyed. An occassional long filament on the surface contained denser masses of material and was distended at the tip.

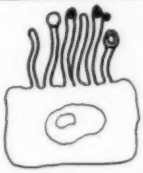
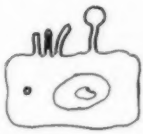
After the embryo had been infected for four days remarkable changes were apparent. Few cells were destroyed. The layer of epithelium was several cells thick, the roughly cuboidal or brick shaped epithelium had become flatter, with one cell overlapping another. Most remarkable were the long filaments projecting into the fluid, sometimes with clusters of spherical particles hung like grapes on their ends (Figs. 14–17). Individual spheres varied in density and it was not possible to tell the occasional "stem" which had been cut in cross section from separate spherical particles. The cells giving rise to these often had normal appearing microvilli at the base, and uniformly the cell's general morphology was well preserved. Lacunae between the flattened cells superficially resembled vacuoles, but few if any true vacuoles were seen in the cell. The mitochondria appeared normal.

Sections of membranes infected for five and six days showed much the same picture with the changes a little more marked (Figs. 18, 19). Hyperplasia was common and the cells were often flattened and overlapping (Fig. 20). In studies on the allantoic fluid of embryos infected with Newcastle virus we had previously failed to find any "filamentous forms", i. e. long rod shaped particles the diameter of the virus. Appearance of these forms both in tissue culture and in sections of the membrane made us look again carefully. This time fresh

* Two embryos each.

allantoic fluid was immediately fixed with either osmic acid or formalin. After varying periods of fixation this fluid was dried on the collodion membrane, washed briefly with water, and then dried. After shadowing with chromium, the specimen was examined in the electron microscope. Unmistakable filaments were present in the allantoic fluid, frequently showing indentations and constrictions (Fig. 24) remarkably like those seen in preparations of preinfected tissue cultures. They were more common (estimated 1 per cent total) in the specimens taken from 5 day vaccine infections, (Fig. 23) but were also pres-

CHART I

	Pre-infected Maximow	Post-infected Maximow	Roller Tube		Sections
			Epith.	Fibr.	
V					
B					
Cg					

Diagrammatic summary of the types of changes produced by the three strains of virus, and as seen with tissue culture techniques and in sections.

ent in "B" infections. Osmium fixation brought out greater density of the central portion of these particles. (Figs. 21, 22).

DISCUSSION

A summary of the findings in regard to the three strains of virus is given diagrammatically in Chart I. The discussion will concern chiefly the virulent (CG) and the vaccine strains. The "chicken avirulent" (B) strain is intermediate between these two and will not be discussed separately. Two main points are involved in this study. The first concerns what the different viruses

do to the host cells and how they get out; the second concerns the nature of the parasitic relationship between virus and host cell.

It seems clear that the virulent strain of virus rapidly destroys individual epithelial cells of the chorio-allantoic membrane. It may also produce within a few hours of infection an erosion of the surface of a cell so that the normal structure is altered. The early destructive effect was best demonstrated in tissue cultures with large amounts of active virus. This strain also invades the cell and causes large clumps of material to appear in the cytoplasm. This may merely reflect the fact that it can use host material deep in the cell. The accompanying destruction of the cell's normal structure thus releases the virus so that it may infect new cells.

The avirulent virus is however, much less destructive for the epithelial cells lining the allantoic sac and indeed seems to stimulate growth. This is reminiscent of the hyperplasia produced by the virus of influenza in mouse lungs (8). Accompanying this hyperplasia there is a marked increase in the number of long surface filaments projecting into the allantoic fluid. The presence of spheres and occasional filaments in the allantoic fluid itself and the appearance of identical units in tissue cultures suggests that these ballooning and disintegrating surface filaments represent the mechanism of virus release. The occurrence of normal surface projections on epithelial cells, both in tissue culture and in sections, then raises the question of their relationship to the virus filament. In cells infected with Newcastle virus, as in cells infected with influenza virus, (9), it seems possible to distinguish virus filaments from normal microvilli. The Newcastle virus filaments are longer than normal microvilli, are usually denser, have frequent variations in density within the filament, and often have swollen tips. The swollen tip may represent an area in which maturation of the virus and osmotic pressure changes together produce the typical ballooning. Since virus filaments so often form on cells which tend to have normal microvilli (epithelium vs fibroblasts) perhaps the virus is able to utilize a normal property of these cells. This agrees in principle with Robinow's theory on the "stalked" forms of influenza virus (10), based on data obtained from vaccinia infections of rabbit cornea.

In dark field studies of the development of the filaments of influenza, Hoyle (11) concluded that the shape of the virus filament was imposed by the surface of the cell. While we agree with this conclusion, we believe that in general conclusions based on optical microscopy at the very limit of resolution (about 100 mμ) are somewhat hazardous.

The prominent and continued alteration of the surface by the avirulent strain focuses attention on this area. Since virus particles are not seen deep in the cell, and since the cell survives in the presence of large amounts of virus at the surface, it is conceivable that virus multiplies in the surface area. Both tissue cultures and thin sections of epithelial cells support this concept. The

avirulent strain, then, seems to limit its growth activities to the surface area of epithelial cells. This surface area probably differs physiologically from the deeper cytoplasm (12), and has been spoken of as the ectoplasm.

It is very likely that the virulent strain multiplies throughout the host cell, for large cytoplasmic inclusions are formed and masses of apparent virus are found therein. The significance of the destruction of the nucleolus is unknown.

If virus escapes through modified microvilli, how much of the individual particle which thus exits from the cell is virus and how much is host? These particles, which average 100 mμ in size, are associated with virus activity in both Newcastle and influenza infections (13–14). If virus is released from the cell through the modification and extreme alteration of the host cell's microvilli, the host substance might well become an inseparable part of the "virus particle". This would account for the areas of greater density in virus obtained from allantoic fluid, and would agree with Knight's observation that purified influenza virus contains a large amount of host protein (15).

These areas seem more resistant to the digestive action of long continued osmium fixation, and correspond in size and shape to areas seen in cells infected with the virulent strain. Whether they represent the smaller hemagglutinating units of Newcastle virus recently described (16) is not yet clear. These smaller units were found, by Granoff, Liu, and Henle, to be in highest concentration in the membrane itself.

There is an increasing emphasis on the presence of nuclei in bacterial cells, and the central dense body of the various pox viruses is well recognized. If this latter may be compared to the nuclei of bacterial cells, then may not the dense areas of Newcastle virus be nuclei? Are we indeed dealing with virus nuclei which are parasitic upon the host's cytoplasm? Such a concept would not exclude a partial conversion of the host's cytoplasm so that the virus "unit" contained a mixture of host and virus cytoplasm.

SUMMARY

Three strains of Newcastle disease virus representing a wide variation in virulence have been studied in thin sections of the chorio-allantoic membrane. It appears that the virulent strain rapidly destroys host cytoplasm, both at the surface and deep within the cell. Apparent virus particles are found there.

The less virulent strain destroys few cells, causes hyperplasia, and seems to be continually present in increasing amounts in the surface layer of the cells. As in tissue cultures, this strain is manifest by a profusion of long filaments which seem to be modified microvilli. These may balloon at the tip and break open, or may break off in clusters of particles. The avirulent form of the virus is thereby released from the cell without causing cell destruction. Forms similar to those seen in tissue culture and in sections are found in infected allantoic

fluid. It is suggested that the infectious "virus particles" thus released may not be free of host cytoplasm.

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PLATE I

Effect of virulent (CG179) strain of NDV on epithelial cells lining the allantoic sac.

Fig. 1. Masses of dense particles deep in cytoplasm. Some of the vesicles may be distended mitochondria. ×12,000.

Fig. 2. Destroyed cell with dense inclusions in cell with clear areas. Adjacent is an apparently normal cell with normal microvilli. ×12,000.

Fig. 3. Destroyed nucleolus within relatively intact nucleus. ×12,000.

Fig. 4. Scattered small dense particles through cytoplasm of infected epithelial cell. Largest ones are about 120 mμ. Average is 50 to 60 mμ. ×12,000

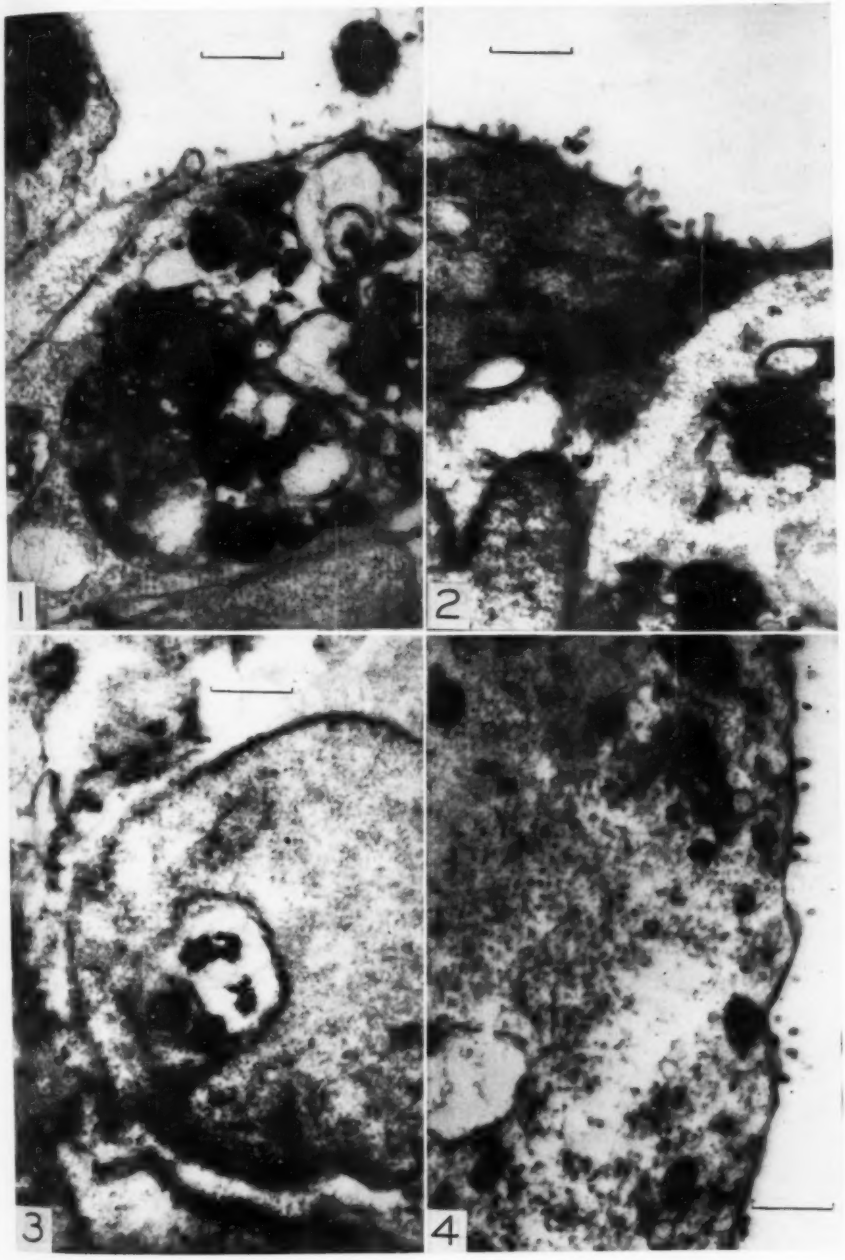
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PLATE II

Disintegration of microvilli and cell surface in infected cells (CG179).

FIG. 5. Microvilli are swollen, and disintegrating. Rest of cell normal. Dense sharply outlined mass in right center is a crystalline artifact. ×8,600

FIG. 6. Effect of large doses of virus. Cell surface is irregular. Mitochondria seem to have been extruded and fine precipitate is deposited on cell surface. Virus had been added 7½ hours before fixation. See text. ×16,300

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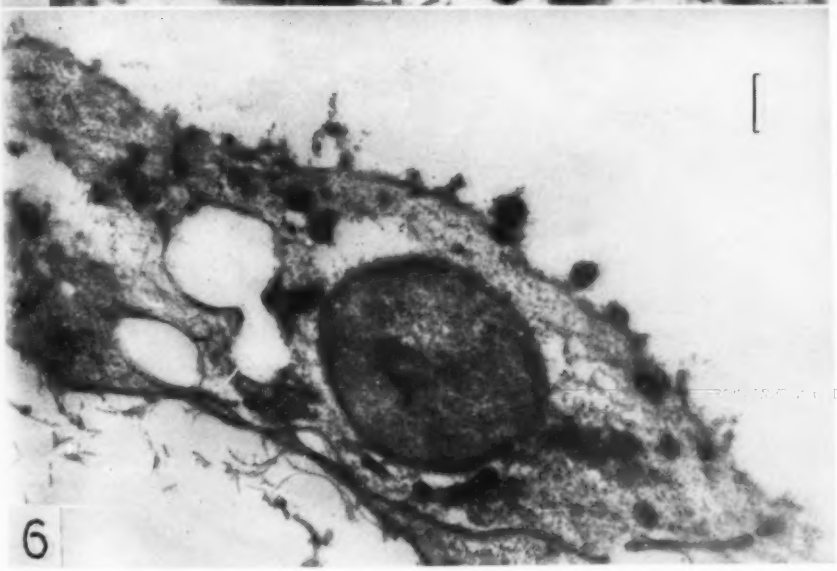

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PLATE III

Effect of intermediate "B" (avirulent for chickens) on epithelial cells.

FIG. 7. Virus particles buried in exudate on top of chorionic cell. Chorio-allantoic inoculation. ×12,000.

FIG. 8. Similar type of preparation. Virus particles in and on surface layer of cell. ×12,000.

FIG. 9. Profuse filaments extending into allantoic sac. Upper cell overlaps lower one. No destruction of cell substance. ×12,000.

FIG. 10. Profuse filament and sac formation on surface of epithelial cell of allantoic layer. ×11,200

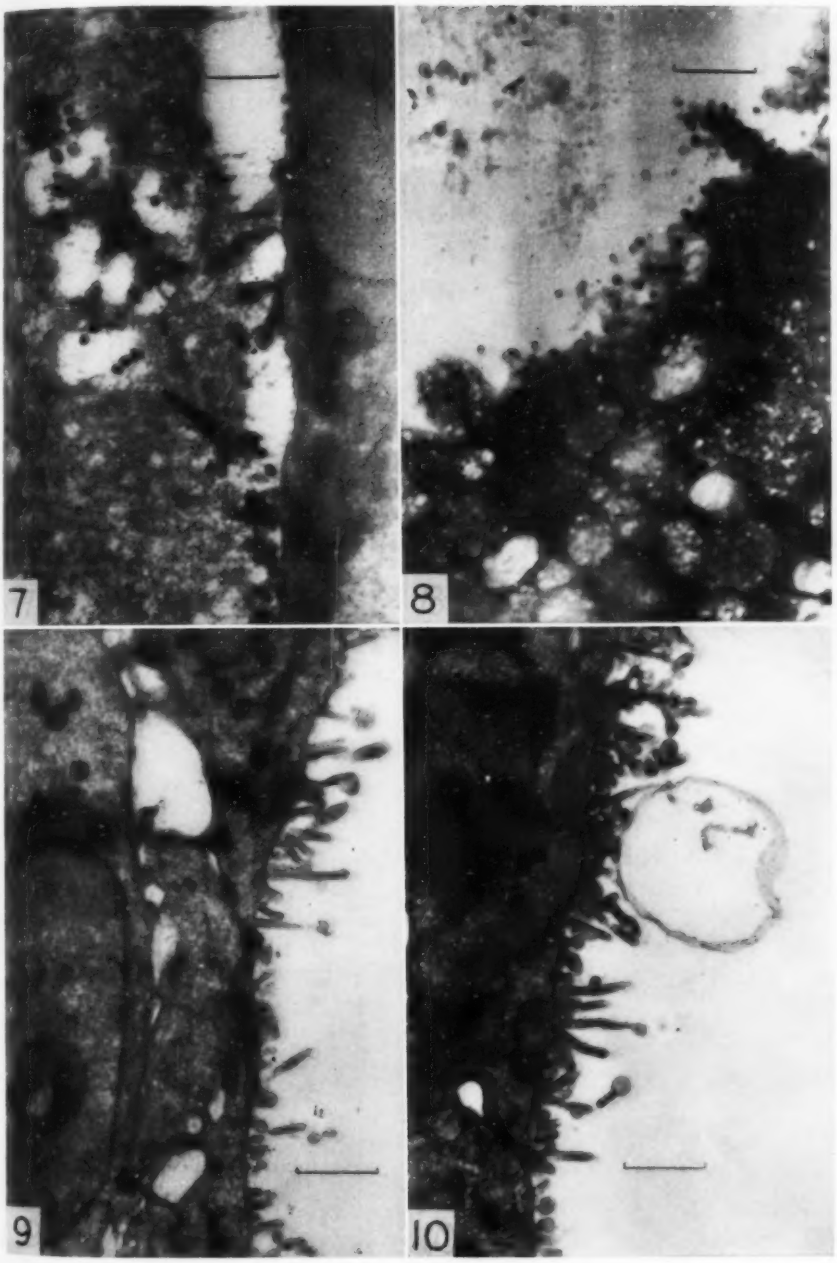
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PLATE IV

Cellular destruction produced by strain of intermediate virulence (B).

FIG. 11. Beneath the surface layer with its filament formation is a large degenerating mass which may have been nucleus. Rest of cell intact. ×12,000.

FIG. 12. Endothelial cell lining blood vessel. Virus has caused distended abnormal mitochondria with spherical masses. Red cells in lower right. Endothelial cells show overlap of several cells. Thin layer of cytoplasm at arrow. ×12,000.

FIG. 13. Destroyed cell with large inclusions in cytoplasm. Distended microvilli at surface. ×12,000.

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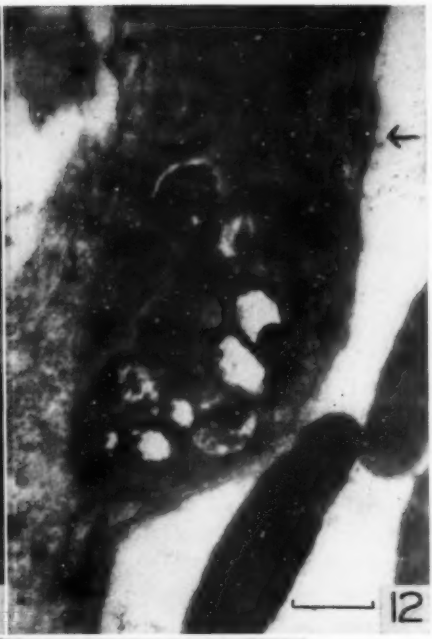
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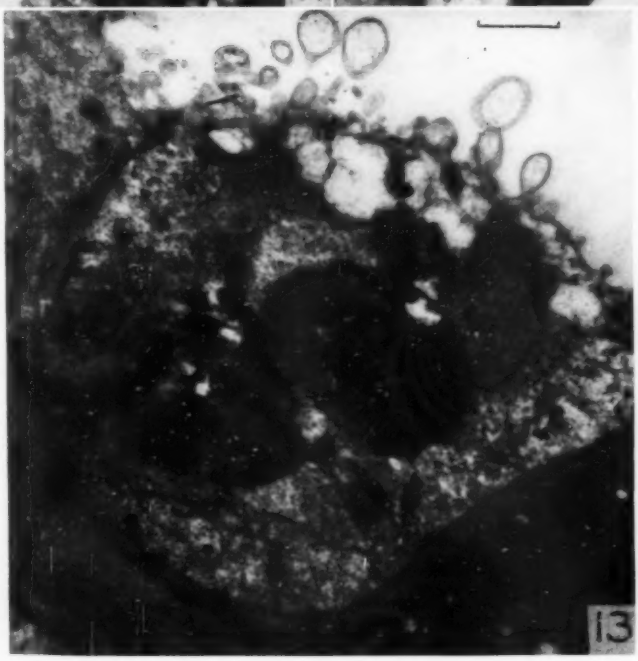
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PLATE V

Effect of vaccine strain on epithelial cells. Four day old infection.

FIG. 14. Long thin virus filaments extending into allantoic fluid. Virus particles breaking off these filaments. ×12,000.

FIG. 15. Fungating mass of virus or altered microvilli. Within tips may be seen dense shell similar to tissue culture preparations. (Figs. 3–4). ×12,000.

FIG. 16. Cluster of filaments with dense virus particles at ends. Cell body normal ×12,000.

FIG. 17. Similar to Figure 16. Nucleus and mitochondria appear normal. ×12,000.

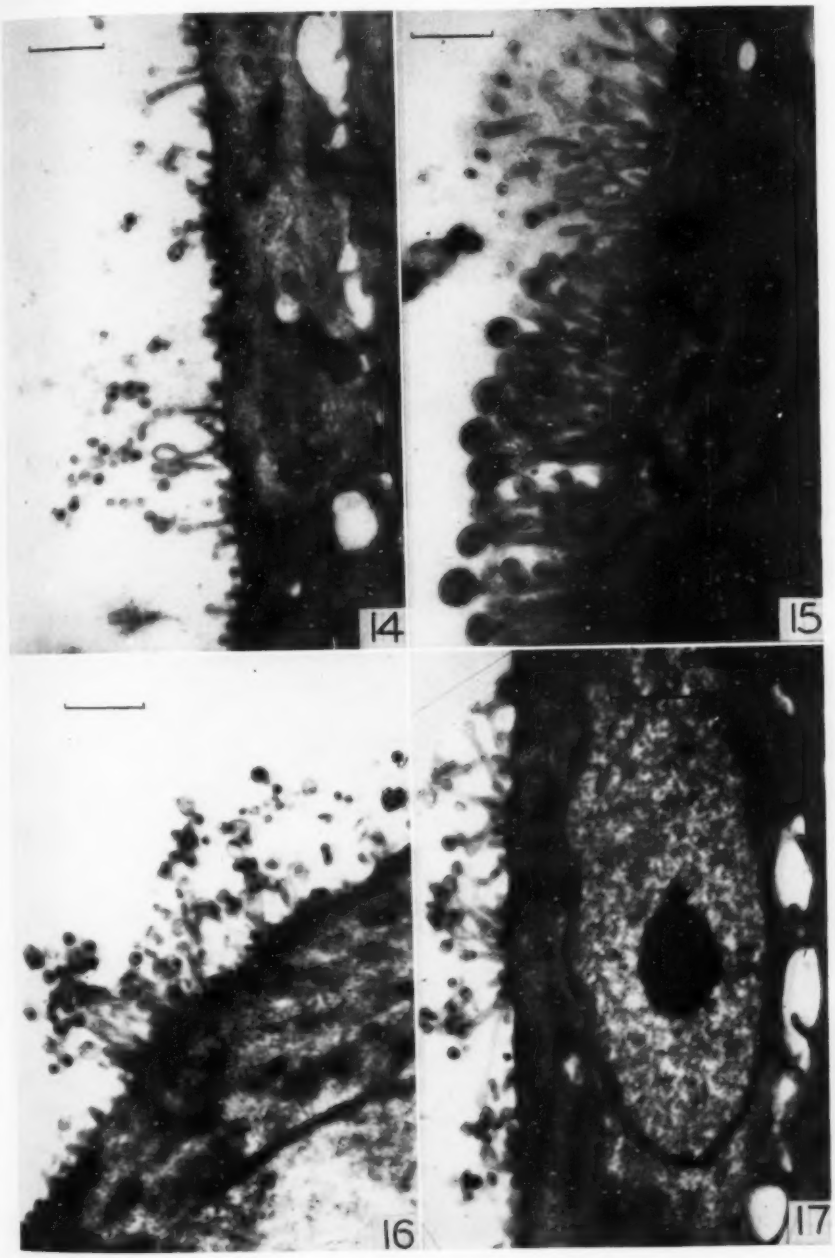
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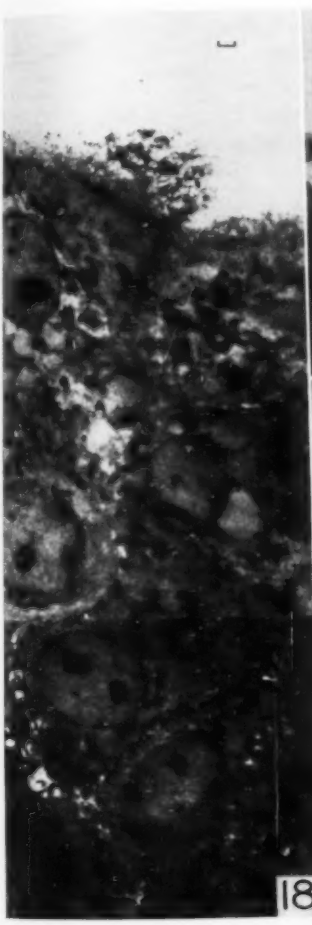
PLATE VI

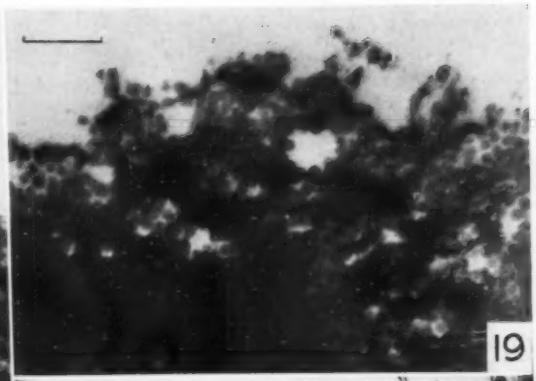
Hyperplasia of allantoic cells produced in five day old infection with vaccine strain of NDV.

FIG. 18. Allantoic layer four or five cells thick. Surface cells covered with virus. Cell in center slightly vacuolated. ×2,050.

FIG. 19. Enlargement of surface of Figure 18. Mass of virus particles and filaments. ×12,000.

FIG. 20. Flattened epithelial cells with heavy surface filament proliferation. Mitochondria normal. ×4,600.





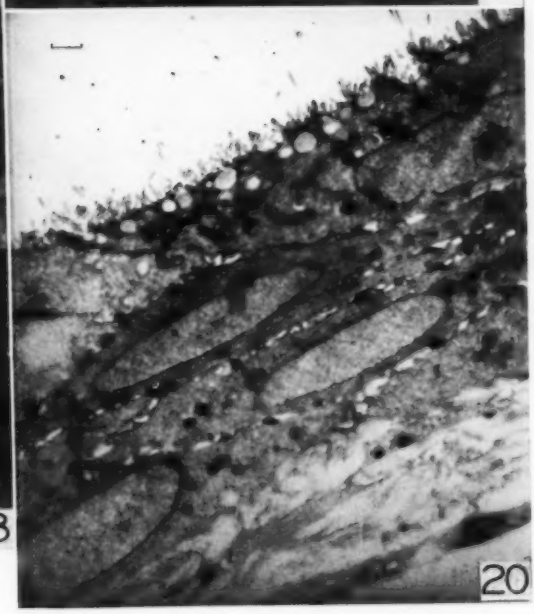


PLATE VII

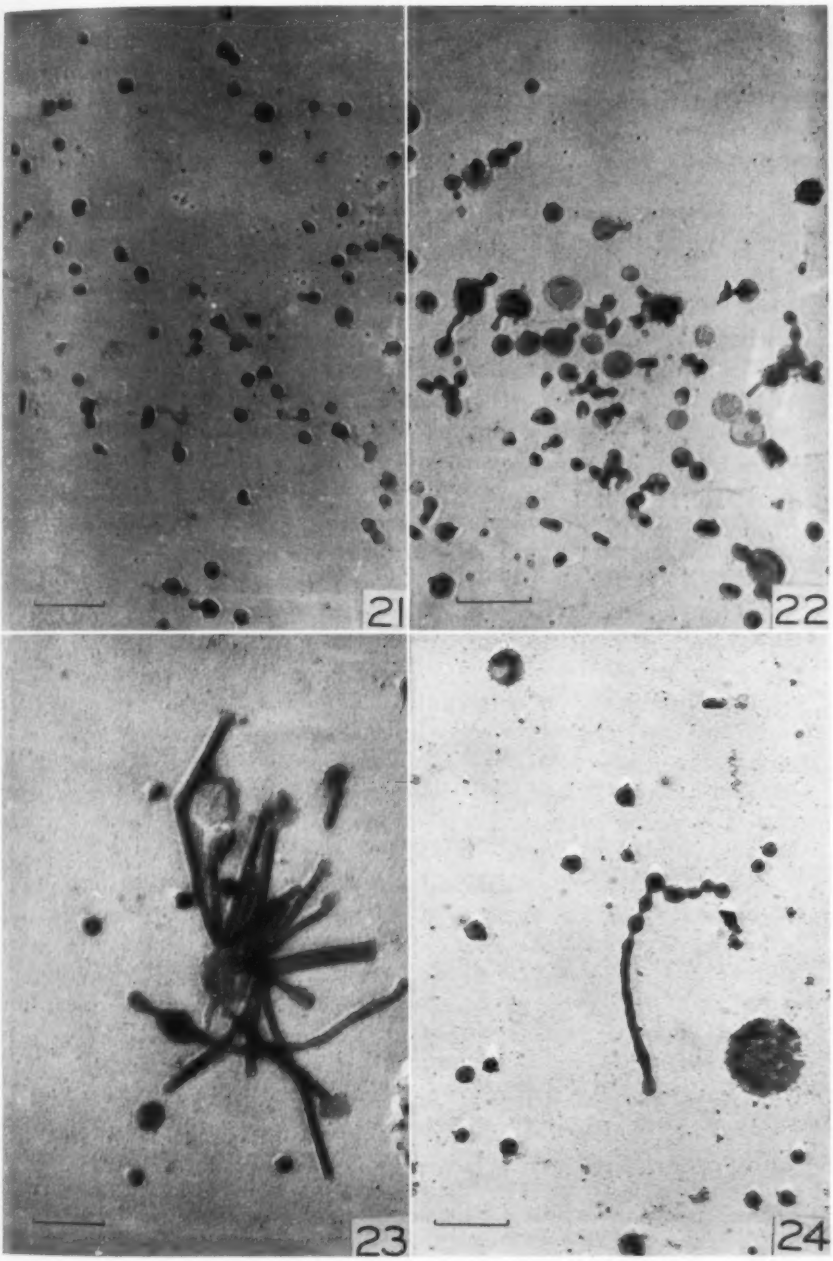
Allantoic fluid with virus.

FIG. 21. Allantoic fluid from embryo infected with B strain for 48 hours. Fixed with formalin, shadowed with chromium. ×11,000.

FIG. 22. Similar preparation but fixed with 1 per cent osmic acid solution. Note dense bodies within particles. ×11,000.

FIG. 23. Cluster of filaments from allantoic fluid preparation of 6 day vaccine strain. Osmic acid. ×11,000.

FIG. 24. Filament with end broken down into individual virus particles. Vaccine strain—6 day infection. ×11,000.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SPLENIC-PORTAL VENOGRAPHY

A Technique Utilizing Percutaneous Injection of Radiopaque Material into the Spleen

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INTRODUCTION

Portal hypertension may result from either intrahepatic or extrahepatic obstruction. Intrahepatic obstruction can usually, but not always, be diagnosed by routine clinical studies as cirrhosis is the most common cause. The presence and site of extrahepatic obstruction cannot be so easily determined by available clinical methods, and in fact determination is difficult even at the time of exploratory laparotomy. The form and extent of extrahepatic obstruction is variable. There may, for example, be a thrombosis of short segments or the major portions of the splenic and portal veins with the vein being converted into a fibrous cord, or there may be a cavernomatous transformation of the vein. A localized stricture of the portal vein has been described infrequently.

The surgical treatment of portal hypertension as practiced today has to do with measures designed to relieve obstruction in the portal venous system. Depending upon the site and type of obstruction, an anastomosis may be performed between the splenic and renal veins, the inferior vena cava and portal vein, or one of the other major branches of each of these structures. In some instances no shunt operation is possible and splenectomy alone is advisable. It is therefore important in planning the surgical attack in any given case of portal hypertension to determine the site of obstruction and the condition of the venous tributaries in the portal system. While considerable information can be obtained during laparotomy by measurement of venous pressures at various sites in the portal system, this is not always adequate. For this reason Blakemore initially, and subsequently others (1–5), have attempted to secure visualization of the involved venous pathways by performing portal venography during laparotomy. Most of these procedures have consisted of cannulating a branch of the portal system, frequently one of the mesenteric veins, followed by the injection of a radiopaque material and the recording of its image radiographically. It is advantageous, however, to have some method of outlining the major branches of the portal venous system prior to the time of laparotomy.

The possibility of such a method of contrast vascular visualization of the portal system became apparent in 1942. At that time a patient was seen with a

chronic draining sinus tract in the left upper quadrant of the abdomen. History revealed that following septicemia in 1936 there had been an empyema, hepatic abscess and multiple recurring abdominal abscesses usually located in the left side of the abdomen. In an attempt to outline the extent of the sinus tract it was injected with 20 cc. of iodochlorol. This revealed in addition to several

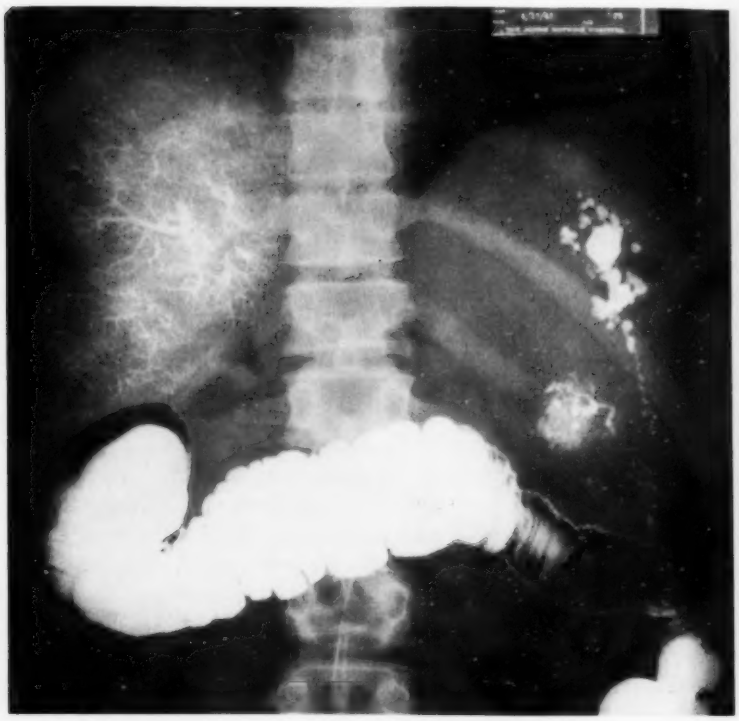

FIG. 1a

FIG. 1. Patient with recurring abscesses and sinus in left upper quadrant.

a. Film soon after injection of sinus with iodochlorol. The hepatic venous system was immediately opacified.

b. Film showing persistence of iodochlorol in the liver nine days after injection.

ramifications of the sinus tract in the left upper quadrant a considerable concentration of the opaque material distributed within venous channels throughout the liver (Fig. 1 a). The exact manner in which the opaque material had reached the portal hepatic branches was never clearly established, but it was presumed that it had entered via rupture into the splenic pulp or gastroepiploic veins. A film nine days later showed persistence of the iodochlorol in the hepatic

area (Fig. 1 b). As far as could be determined the patient suffered no ill effects from this accidental introduction of radiopaque substance into the portal circulation.

Intermittently during the next few years experimental and clinical efforts were made to duplicate this opacification of the portal system. Recent reports

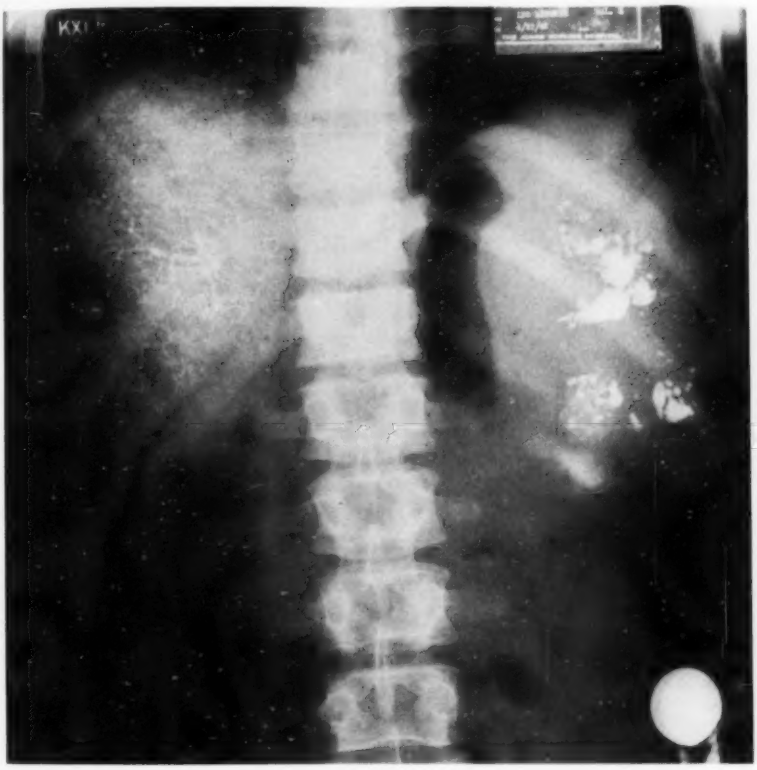

FIG. 1b

concerning the work of others have prompted us to record our experiences at this time (5, 6, 7, 8).

EXPERIMENTAL AND CLINICAL EXPERIENCE

In 1949 experiments were started on dogs in which various contrast media were injected into the splenic pulp, under direct vision at laparotomy, and serial radiographs were obtained during and following injection. Experience quickly showed that 70 per cent Diodrast was an acceptable contrast substance,

and it was found that following the rapid injection of this into the splenic pulp there was almost immediate opacification of the splenic vein. Within two or three seconds the intrahepatic portal branches were well filled in most instances (Fig. 2, a and b), although in some instances the branches of the left lobe of the liver did not receive a significant amount of the opaque medium.

FIG. 2a

FIG. 2. a. & b. Venograms exposed at end of injection of 70 per cent Diodrast into splenic pulp of dog. Note immediate filling of normal splenic and portal veins including intrahepatic branches.

This selective filling of the intrahepatic portal branches to the right lobe is not understood and is somewhat contrary to the "streamlining" of splenic blood to the left lobe reported by others (9, 10). After approximately five seconds the opacification of the portal system usually began to decrease rapidly, although a small amount of residual opaque material lingered in the spleen for a period of twenty to thirty minutes. Examination of the spleen of such dogs at intervals following injection showed small infarcts in various stages of healing.

Following the successful demonstration of the portal system in animals, clinical application of the technique was made. Between 1949 and 1952 six patients were studied. In brief, the method employed in these clinical studies was as follows. The patient was placed in a supine position on a radiographic table and under general or local anesthesia an 18 gauge needle, two and a half

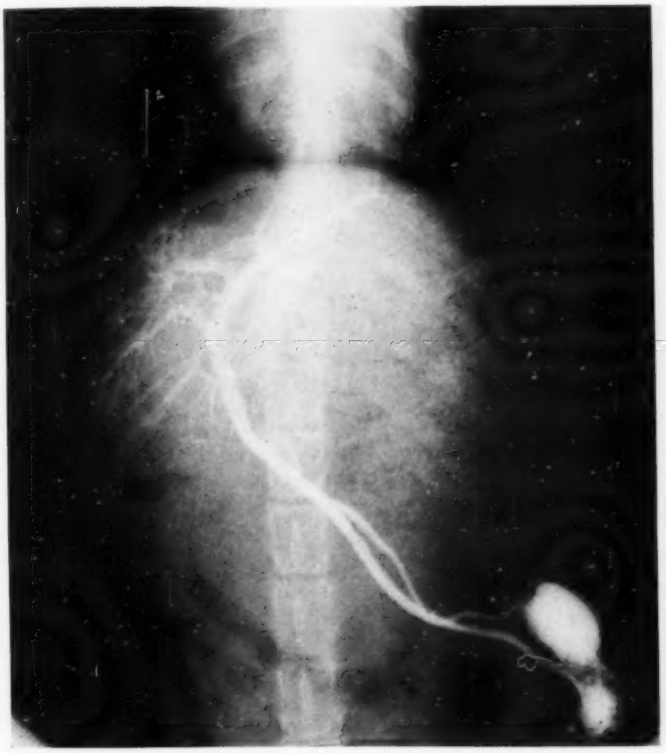

FIG. 2b

inches long, fitted to a standard 20 cc. or 50 cc. syringe was introduced percutaneously into the spleen. Experience revealed that it was most satisfactory to introduce the needle obliquely into the body of the spleen, rather than to attempt to follow the long axis of the viscus. Frequently, but not always, blood could be aspirated when the needle was satisfactorily positioned within the splenic pulp. A solution of 70 per cent Diodrast was then injected as rapidly as possible, the amounts ranging from 12 cc. to 40 cc. depending upon the size of the patient. In most instances the injection was completed within 2 to 3 seconds. Serial films at the rate of one per second, starting toward the end of the

injection, or a single film at the completion of the injection were obtained in all instances.

Our experiences with the technique are presented in the following brief case histories.

Case 1.

K. B., a five year old white girl was admitted to the hospital in 1949 because of splenomegaly and hematemesis which had occurred on three occasions during the preceding seven weeks. On examination the spleen was enlarged and extended almost to the umbilicus. The liver was not felt. The blood contained 12 grams of hemoglobin; tests of liver function were normal. Esophageal varices were demonstrated radiologically. Splenic-portal venography was attempted under local infiltration anesthesia, twelve milliliters of 70 per cent Diodrast being injected. The patient immediately complained of pain in the left shoulder and left upper abdomen which lasted about ten minutes. The film taken at completion of the rapid injection showed that most of the Diodrast had been injected beneath the splenic capsule. In spite of this the splenic vein could be visualized faintly. Splenic-portal venography was repeated without success two and a half weeks later, this time under general anesthesia just prior to operation. Almost all of the Diodrast was mistakenly injected outside the spleen. At laparotomy there was some free fluid, much of it in the region of the splenic hilum. The tail of the pancreas was more than usually adherent to the spleen. The portal vein appeared normal. Splenectomy and splenorenal venous anastomosis were performed. The spleen showed evidence of portal obstruction and a well localized infarct at the site of previous injection. Bleeding into the gastro-intestinal tract occurred once during her hospital stay. After three years of apparent good health she had another hematemesis from which she recovered.

Comment: Visualization of the splenic vein following even subcapsular injection of Diodrast suggested clinical usefulness and further trial of the method. Splenic puncture in both instances was made in the direction of the long axis of the spleen. An oblique puncture across the body of the spleen has since been more satisfactory. We were encouraged by the absence of complications when Diodrast leaked into the peritoneal cavity.

Case 2.

T. L. B., a 14 year old male was admitted because of anemia and leukopenia discovered during a febrile illness. Splenic and hepatic enlargement had been present for many years. On examination the liver edge could be percussed two fingerbreadths below the costal margin and a large spleen was felt extending into the left lower quadrant. Routine studies revealed no definite cause for the splenomegaly or hepatomegaly. No esophageal varices could be demonstrated, and lymph node biopsy showed only hyperplasia. Splenectomy was advised because of the size of the structure and for diagnosis.

Splenic-portal venography was done under local anesthesia just prior to operation in an effort to rule out splenic venous thrombosis as a cause of splenomegaly. After percutaneous puncture of the spleen blood could be aspirated but not readily nor from all areas. Forty milliliters of 70 per cent Diodrast were injected rapidly, and a film was exposed when almost all of the Diodrast had been injected (Fig. 3). The immediate filling of normal splenic and portal veins was seen. There was no evidence of obstruction. The portal tributary to the left

lobe of the liver was only faintly outlined. At laparotomy there were 30 to 40 milliters of bloody fluid in the peritoneal cavity, but needle holes were barely visible in the spleen. Splenectomy was performed and the patient has remained well since discharge from the hospital three years ago. Pathological examination of the spleen showed a small infarct and diffuse fibrosis. A liver biopsy was normal.

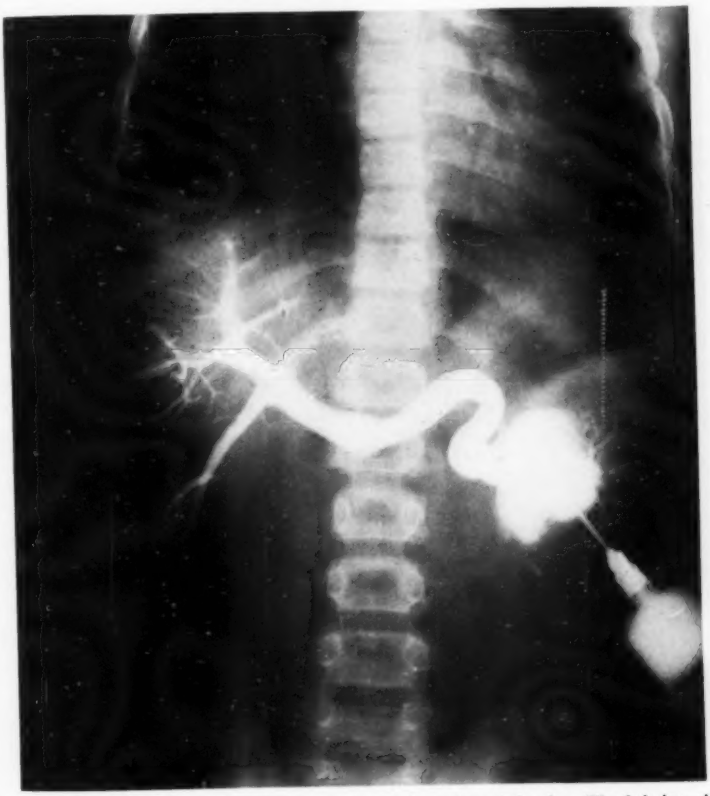

FIG. 3. Case 2. Venogram showing normal splenic and portal veins. The left intrahepatic branch of the portal vein is only faintly opacified.

Comment: Visualization of normal splenic and portal veins without collateral channels ruled out portal venous obstruction and hypertension and the need for a shunt procedure.

Case 3.

R. J., a five year old boy was admitted in 1949 after a single episode of melena and massive hematemesis. A large spleen and liver had been noted two years previously. On examination the liver was felt two fingerbreadths below the costal margin and the edge of the spleen

halfway between umbilicus and costal margin. Except for a two plus cephalin flocculation all chemical examinations of the blood were normal; there were 1.4 million red blood cells per cubic millimeter.

FIG. 4a

FIG. 4. Case 4.

a. Venogram showing large splenic vein in a patient with cirrhosis. Collateral veins running in the direction of the esophagus can be seen. Note angulation and streamlining at junction of splenic and superior mesenteric veins just to right of spine.

b. Venogram exposed 2 seconds after injection of Diodrast. A large collateral vein running transversely to the right of the splenic-portal junction can be seen along with other smaller veins.

Splenic-portal venography was performed by percutaneous injection under ether anesthesia immediately prior to laparotomy, fifteen milliliters of 70 per cent Diodrast being used. Blood could be aspirated from the spleen prior to injection. Due to technical difficulties completely satisfactory films were not obtained. A small splenic vein, many collaterals, and

a large vessel possibly representing the coronary vein could be identified. The portal vein itself could not be seen. On a film taken several seconds later the paravertebral venous plexus was clearly opacified.

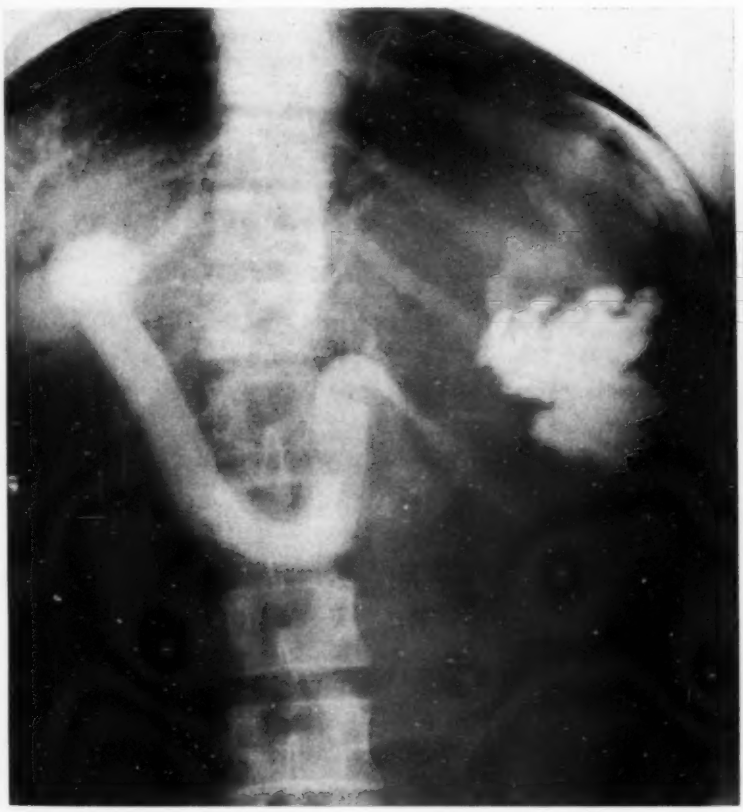

FIG. 4b

At laparotomy there were about 30 milliliters of blood in the peritoneal cavity but no puncture holes could be identified in the spleen. The only splenic vein which could be found was several millimeters in diameter and unsuitable for anastomosis. Splenectomy was performed. Pathological studies revealed a hematoma at the site of injection. The patient was apparently well when last seen.

Comment: Splenic-portal venography failed to demonstrate a large splenic vein and revealed obvious obstruction and extensive collateral circulation. We have not yet studied by this method a patient with a patent portal vein in conjunction with splenic vein thrombosis. We believe that such a portal vein

would be filled with Diodrast. On the basis of the study of this patient we suspected that no portal vein would be found at laparotomy for a portal-caval anastomosis and an extensive dissection was avoided. Simple splenectomy was performed.

Case 4.

M. B., a 47 year old woman was admitted in 1949 because of two recent episodes of hematemesis. There was a history of questionable hematemesis one year previously. Studies elsewhere five months previously had revealed splenic and slight hepatic enlargement, ascites and sacral edema, bromsulfalein retention and roentgenographically demonstrable eosphageal varices. Examination on admission here revealed a liver palpable five to eight centimeters and the spleen palpable seven centimeters below the costal margin. No spider angiomata were noted. There was a systolic bruit in the tricuspid area. Varices extending to the level of the aortic arch were demonstrated on barium swallow. The blood contained 1.4 milligrams per cent of bilirubin. Cephalin flocculation, thymol turbidity, serum albumin and globulin were normal. Obstruction of the splenic vein was thought to be the most likely diagnosis although primary cirrhosis could not be excluded.

Percutaneous splenic-portal venography was done under local infiltration anesthesia, 70 per cent Diodrast being used. Large splenic and portal veins were opacified on a film taken just at the end of injection (Fig. 4 a). In a film taken two seconds later a large vessel thought to be a collateral vein could be seen running transversely in the right upper quadrant (Fig. 4 b). A diagnosis of cirrhosis was made and this was confirmed at laparotomy. The large splenic vein was anastomosed to the side of the left renal vein. The patient has remained well following convalescence.

Comment: On the basis of this study done without difficulty under local anesthesia the diagnosis of cirrhosis was established. It was apparent that either portal or splenic vein was of adequate size for anastomosis.

Case 5.

E. M. H., a 34 year old woman was admitted in October 1950, one day after a massive hematemesis and melena. Studies in the Medical Out Patient Clinic seven months previously had revealed splenomegaly and elevated leucocyte and platelet counts. On the basis of these findings and a sternal marrow biopsy a tentative diagnosis of "agnogenic myeloid metaplasia" was made. On admission incipient shock and hypochromic anemia were treated with numerous transfusions of whole blood. Esophageal varices were demonstrated radiographically.

Splenic-portal venography by percutaneous injection revealed what appeared to be a splenic vein about 1 centimeter in diameter (Fig. 5 a). Opacification was mottled and borders were irregular. Numerous and extensive collateral channels were opacified (Fig. 5 b). The portal vein could not be identified and there was delayed filling of intrahepatic portal branches.

Laparotomy immediately after this study revealed free blood in the peritoneal cavity. Needle holes could be seen in the spleen but there was no bleeding at the time. Splenectomy was performed. There was cavernomatous transformation of the splenic vein. Large collaterals but no portal vein could be identified in the porta hepatis.

After operation thrombosis and pulmonary embolism occurred. Superficial femoral venous ligation was performed bilaterally but she died twenty days following splenectomy of mas-

FIG. 5a

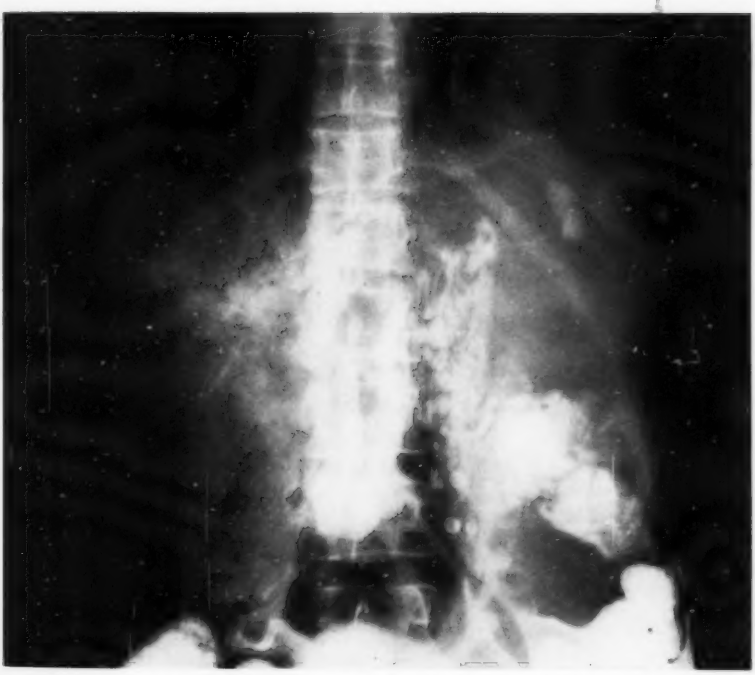

FIG. 5b

FIG. 5. Case 5. A patient who had cavernomatous transformation of the splenic and portal veins.

a. Venogram showing only short segment of small splenic vein. The edges are irregular and opacification is mottled.

b. Film exposed later shows extensive collateral circulation. The portal vein cannot be identified and filling of intrahepatic branches was delayed.

sive pulmonary embolism, thought to have arisen from the femoral and iliac veins above the point of ligation. There was cavernomatous transformation of the portal and remaining splenic veins. There was no explanation for the thrombocytosis.

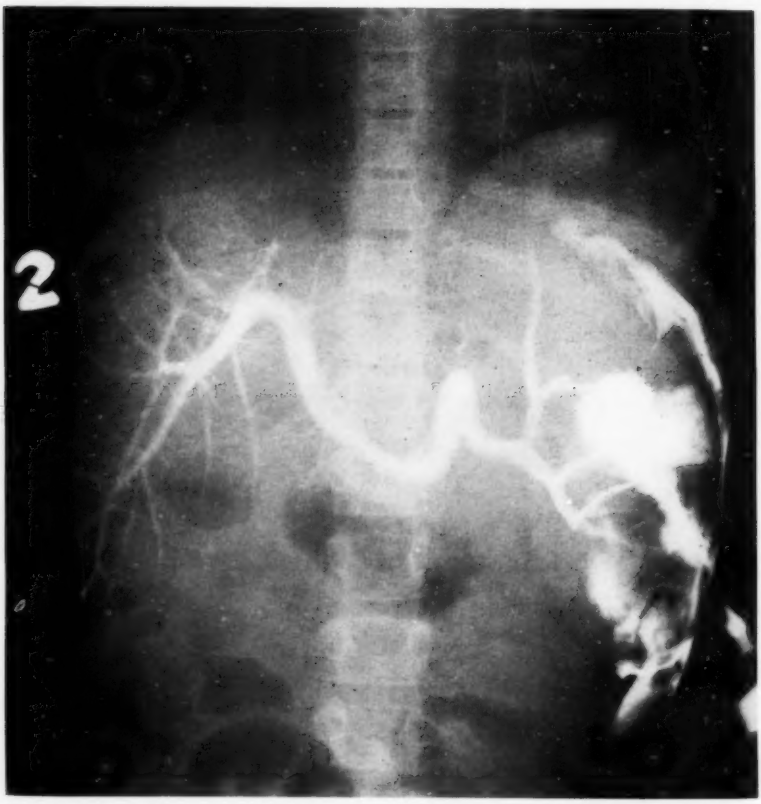

FIG. 6. Case 6. Venogram showing normal splenic and portal veins in a patient with biliary cirrhosis. Note Diodrast which has leaked around periphery of large spleen. The left intrahepatic branch of the portal vein is only faintly opacified. Portal hypertension was demonstrated by measurement of pressure.

Comment: Splenic-portal venogram revealed an obvious obstruction and extensive collateral circulation. The mottled and irregular appearance of the splenic vein and delayed filling of intrahepatic portal branches suggested cavernomatous transformation. Useless and difficult dissection in search of a vein for anastomosis to the renal vein or inferior vena cava was avoided on the basis of the contrast study.

Case 6.

N. M., a ten year old girl, was admitted to Harriet Lane Home in August, 1952, because of jaundice and ascites. In 1949 she was explored elsewhere because of vomiting and jaundice, and a bile-containing cyst was found in the right upper quadrant of the abdomen. This was initially drained and then later anastomosed to the duodenum, the diagnosis being choledochal cyst. Her post-operative course was not satisfactory. In 1950 she had massive abdominal swelling, intermittent fever of 104° F, light colored stools and dark urine. She was again hospitalized for three months, following which she did well until July 1952 when abdominal swelling again appeared, along with nausea, light colored stools and dark urine.

On admission to this hospital she was cachectic and chronically ill. The liver and spleen were felt seven to ten centimeters below the costal margin, and massive ascites was present. Moderate edema of the lower extremities was noted.

Chemical examinations of the serum showed an alkaline phosphatase of 37.4 units, two plus cephalin flocculation, thymol turbidity of 9.5 units, albumin-globulin ratio of 2.8/4.4 gm. per cent, and bilirubin of 2.8 mgms. per cent.

Barium swallow showed no evidence of varices. Gastro-intestinal series showed regurgitation of barium into the biliary system. A tentative diagnosis of intermittent ascending cholangitis was made, but splenic-portal venography was performed to rule out portal thrombosis.

Twenty milliliters of 70 per cent Diodrast were injected into the spleen percutaneously under general anesthesia just prior to laparotomy. The splenic and portal veins were normal and there was no evidence of collateral circulation (Fig. 6).

At laparotomy by Dr. David Sabiston the liver showed considerable biliary cirrhosis. Two enlarged biliary ducts entered the duodenum in the region of the ampulla of Vater where obstruction of one of the ducts could be demonstrated. There was no evidence of the previous anastomosis between duodenum and biliary system. Because of the anatomical anomaly and entrance of oral barium into the biliary system, intermittent obstruction and cholangitis seemed to be the cause of biliary cirrhosis. The two large biliary ducts were anastomosed to a Roux-Y loop of jejunum. Pressure was measured in the superior mesenteric vein and found to be 350 mm. of saline.

Her post-operative course was free of complication and she was discharged improved.

Comment: Splenic-portal venography in this instance excluded portal thrombosis as a cause of the patient's ascites.

DISCUSSION

Portal hypertension may result from either intrahepatic or extrahepatic obstruction. Intrahepatic obstruction is usually due to cirrhosis and can often, but not always, be diagnosed by routine clinical studies. There may occasionally be portal venous thrombosis associated with cirrhosis. Extrahepatic obstruction may be located in the portal vein itself or in one of the major tributaries of the portal vein such as the splenic. The obstruction may extend throughout the portal system or be localized to a large or small area. It may be of three types. There may be thrombosis of the splenic or portal vein with a resulting fibrous cord, a cavernomatous transformation of the vein, or finally and less frequently there may be a localized stricture.

It is important to determine the site of obstruction and condition of veins in the portal system in planning a surgical procedure. A time-consuming, difficult dissection can be avoided if this can be done simply before operation. Considerable information can be obtained during laparotomy by measurement of venous pressures at various sites in the portal system. This has been supplemented by Blakemore and others (1–5) by portal venography and contrast visualization of the portal system during laparotomy. Percutaneous splenic-portal venography may furnish such visualization without the difficulties of positioning, exposure and delay during operation.

Percutaneous injection of Diodrast into the spleen for visualization of the portal system has been performed without complications in this small series of patients. Bleeding, the complication which we most feared, has not been a problem, although most of the examinations were done just prior to laparotomy because of this fear of hemorrhage. Another possible objection to the method is that Diodrast may increase the likelihood of thrombosis of the anastomotic site, but we have no evidence that this occurs. Needless to say it is desirable that the spleen be large enough to be felt easily and injected. However, unless the spleen has been previously removed, splenomegaly is a constant accompaniment of portal hypertension.

SUMMARY

Determination of the site of obstruction in portal hypertension is important in planning operations designed to reduce the pressure in this condition. Intrahepatic obstruction due to cirrhosis can usually be diagnosed by available clinical methods. The site and type of extrahepatic obstruction cannot be determined with similar ease and accuracy.

A technique is described for the preoperative percutaneous injection of radiopaque material into the spleen with resulting contrast visualization of the portal venous system. The results of studies on six patients are described.

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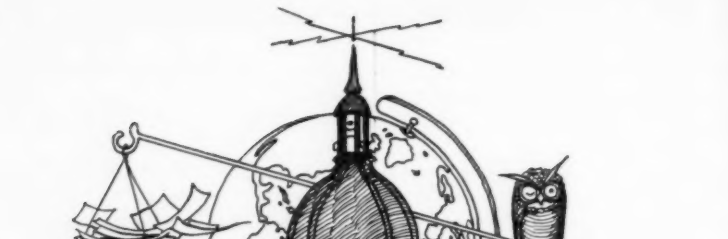

BOOK REVIEW

(These reviews represent the individual opinions of the reviewers and not necessarily those of the members of the Editorial Board of the Bulletin)

Nutrition and Diet in Health and Disease, 6th edition. By JAMES S. MCLESTER AND WILLIAM J. DARBY. 710 pp., $10.00. W. B. Saunders Co., Philadelphia, Pa.

Nutrition and Diet in Health and Disease, was written by two outstanding authorities in the field of nutritin. This book is divided into two parts. Part I, the primary responsibility of the junior author, deals with nutrition in health and Part II, that of the senior author, is devoted to nutrition in diseases.

Specifically, the first two chapters in Part I of this book start with introductory remarks on the utilization of food which includes the digestion and metabolism of proteins, carbohydrates and fats, with due consideration of energy metabolism of the cell. Discussion on the nutritional requirements for proteins, fuels, individual vitamins, inorganic nutrients and nutritional factors of lesser importance follow in Chapters 3 to 7 inclusive. Specific food products such as milk, meat, vegetables and fruits and so forth are covered in Chapters 8 to 10. In the last three chapters of Part I the authors present a resume of the feeding problems of normal individuals of different ages, and of individuals with special feeding problems such as infants as well as women during periods of pregnancy and lactation.

In Part II the nutritional problems in various diseases such as those of single as well as multiple deficiencies of vitamins are presented. Nutritional requirements in different metabolic diseases such as diabetes mellitus, gout, obesity, diseases of the kidney and urinary tract are also well reviewed. In addition, the author also describes nutritional problems in specific diseases involving endocrines, the blood, the heart and arteries as well as nutritional problems after surgery and febrile diseases. An appendix at the end of the book gives special methods of feeding and supplementary tables on the calories and composition of foods which should be most useful to those engaged in practical nutrition. At the end of each chapter the authors provide extensive references including the titles of the articles. This book is therefore highly recommended to those interested in nutrition. However, even with this well written book a few minor comments are in order. First, a reader who is interested in the science of nutrition rather than the practical management might feel a lack of adequate discussion on the cause of the diseases (e.g. chapter on diabetes) and on the functions of

vitamins in enzyme systems. Secondly, in the chapter on milk and milk products it should be equally important to mention that in addition to fats, carbohydrates and proteins, milk provides a number of important nutritional factors, present both in the proteins and in the whey.

BACON F. CHOW

Textbook of Surgery. Edited by H. F. MOSELEY. 896 pp., $15.00. The C. V. Mosby Co., St. Louis, Mo.

This is a new textbook of surgery prepared by twenty-eight faculty members of the McGill University School of Medicine and the Royal Victoria Hospital. The striking features of this book are its conciseness, clarity, and completeness. Although there are multiple authors, the literary style and organization is consistent and each chapter is quite readable. The first chapter is entitled "The Evolution of Modern Surgery". Here is presented the course of the art and science of surgery from the Neolithic Age, through the early Egyptian records of 3000 B.C., the Indian Rig-Veda of 1500 B.C., and the eras of Hippocrates, Celsus, and Galen. The contributions of Vesalius, Paré, Harvey, and John Hunter are considered. Important features of the introduction of anesthesia and the Listerian era are discussed, and the modern advances are included in the division headed "Surgery in the Twentieth Century". This outline of the history of surgery should be of considerable help in orienting the student in the progress of surgery.

The basic chapters, which include "Reaction to Injury and Repair", "Surgical Bacteriology and Chemotherapy", "Shock and Blood Transfusion", and "Anesthesia" are all written by those who are obviously specialists in these fields. The section devoted to thoracic surgery is unusually well-organized. The various traumatic lesions and their treatment are considered. The newer concept of the segmental anatomy of the lung and its surgical importance is clearly set forth including illustrations. A subject which is frequently difficult for the student to grasp, that of the surgery of pulmonary tuberculosis, is quite readable and commendable.

In keeping with recent advances a chapter is devoted to the surgery of cardiac disorders. Since this is a relatively new subject in textbooks, the contents of this chapter will be considered here in more detail. The field is well covered and includes acute cardiac tamponade, patent ductus arteriosus, coarctation of the aorta, auricular and ventricular septal defects, tetralogy of Fallot, pulmonic stenosis, tricuspid atresia, transposition of the great vessels, truncus arteriosus, mitral stenosis, and coronary artery disease. In regard to therapy in the various disorders, pericardial aspiration is considered the treatment of choice for acute tamponade. For obliteration of the patent ductus arteriosus, multiple ligation is considered the safer procedure, while that of division "is more hazardous, but insures complete and permanent occlusion of the ductus". For the tetralogy of Fallot the subclavian-pulmonary artery anastomosis is favored as being "probably the most satisfactory method". Importance is laid upon the differential diagnosis of the tetralogy of Fallot and isolated pulmonic valvular stenosis, inasmuch as the only satisfactory treatment for the latter is pulmonic valvulotomy. The experimental and clinical investigation on surgical procedures for the relief of coronary insufficiency is critically evaluated. The closing remarks are highly sound and warrant quoting: "To date, all operations for coronary disease have carried a high mortality, and in none have the results been convincingly beneficial. The whole subject requires a great deal more experimental work before a wide application to human material can be justified".

There are many other excellent chapters including "The Stomach, Duodenum, and Small Intestine" which is particularly well-illustrated, and the chapter on the "Liver" which includes a clear discussion of jaundice with detailed consideration of the tests of liver function.

The specialties of plastic surgery, neurosurgery, urology, and orthopedics are covered in individual chapters by members of their respective departments.

This new work, which contains a number of references published in the year of its publication, is unquestionably abreast of the field. It can be recommended for students during their courses in surgery as a text which adequately and commendably covers the subject and does it within the limits of a volume of moderate size.

DAVID C. SABISTON, JR.

Grundriss der Elektrokymographie. Phasenanalyse und Raumkymographie des Herzens. By K. HECKMANN. 36 pp., Kart. DM 6.60. Georg Thieme Verlag, Stuttgart, Germany.

The author, a colleague of Pleikart Stumpf of Munich, like him has had many years of experience with roentgenkymography as background for his study of electrokymography. This little handbook is well illustrated by simple line drawings. In the first part, nine normal and thirty-one abnormal EKY patterns are presented. (For timing purposes Heckmann used the phonocardiogram superimposed on the EKY, i.e., recorded by the same galvanometer.) The second part of the booklet deals with what the author terms "phase analysis". He emphasizes that in the case of most ventricular border tracings, normality or abnormality depends, of course on what segment of the border is traced, but particularly on what the pattern is elsewhere over the ventricular silhouette. His phase analyses consist of comparisons of temporally corresponding portions of tracings from several sites on the heart border. By means of these analyses, he defines some normal and abnormal patterns of movement of the heart—rotation, changes in shape, pendulum movement, and so on—other than those movements involved directly in ventricular filling and emptying. Recently, books on ballistocardiography and on vector-cardiography have appeared but this is probably the first on electrokymography. It should have definite, though restricted, usefulness to those working with electrokymography and perhaps in some closely related fields such as ballistocardiography.

VICTOR A. MCKUSICK

Lehrbuch der Röntgenologischen Differentialdiagnostik. Vol. 1, 3rd edition. By WERNER TESCHENDORF. 954 pp., Ganzleinen DM 129 (about $32.00) Georg Thieme Verlag, Stuttgart, Germany.

This compendious work is an attempt at a systematic approach to x-ray differential diagnosis of the thoracic organs. Its organization is a morphologic one. For example, the author considers all entities which cause obscuring of an entire hemithorax, with increase or decrease in the volume of the affected side. Again, there is treatment of the causes of lobar densities, of densities in individual small portions of the lungs within a given lobe. These are subdivided according to the x-ray appearance—round and triangular densities, those with well defined and poorly defined margins. There are sections devoted to various types of mottling of the lung fields, to cavitation and cyst formation and to the normal lung markings. The pleura, hila and mediastinum receive individual attention.

In addition to the section on the lungs, there are three other divisions dealing with heart, diaphragm and esophagus. As might be expected, the cardiac section is less well suited to a morphologic approach, and the discussion is principally on an etiologic basis, although the early sections deal extensively with the normal variations in heart size and shape and with x-ray methods for evaluating these. Many conditions, such as disturbances of conduction, myocarditis, and coronary artery disease, in which there is no characteristic x-ray pattern, or in which the x-ray contributes only confirmatory evidence, are given too much emphasis.

It is felt that this work may be criticized more generally for the rather too minute analyses of extremely rare conditions. For example, there is a one-page chart devoted to pulmonary

syphilis and its x-ray diagnosis. There has been extensive recourse to the literature by the author, and the text overlooks almost no described entity or process. The illustrations are of good quality.

This work is a sort of radiologic "French's Differential Diagnosis". It is regarded by the reviewer as a reference book for the medical library or for the book-shelf of the radiologist who can read German. It is an encyclopedia of case-radiographs indexed as much as possible according to their x-ray morphology, with accompanying descriptive text.

DANIEL TORRANCE

Die Angiographie zur Erkennung, Behandlung und Begutachtung peripherer Durchblulungsstöneugen. By HABIL H. PÄSSLER. 115 pp., Kart. DM 29.70 (about $7.50). Georg Thieme Verlag, Stuttgart, Germany.

This is a concise yet comprehensive monograph dealing with all aspects of peripheral vascular diseases of the legs, but with special emphasis on angiography (femoral arteriography, aortography and venography) in diagnosis and treatment.

The author commences with a section on the physiology and pathology of arterial insufficiency. He discusses Raynaud's disease, then divides the organic types into endangitis obliterans and arterio-, and atherosclerosis.

He next discusses the various methods of evaluating the degree of insufficiency. In his own work he relies heavily on the oscillograph and on angiography.

Then follows a discussion of the techniques of arteriography, aortography and venography. There is good treatment of the importance of timing in relation to the degree of insufficiency in the taking of serial arteriograms.

The final sections deal with treatment and prevention.

The author writes clearly and with the authority of clinical experience. His illustrations and case presentations are well correlated. His correlation of all the aspects of the important problem of peripheral vascular disease removes this desirable monograph from the radiologic literature alone and makes it of first-rank importance to surgeon, internist and radiologist.

DANIEL TORRANCE

Operating Room Technic. St. Mary's Hospital, Rochester, Minnesota, 4th edition. By SISTERS OF ST. FRANCIS OF ST. MARY'S HOSPITAL. 345 pp., W. B. Saunders Co., Philadelphia, Pa.

Although the primary objective of this compact hand book and work book is designed to meet specific needs in the care of operative surgical patients at St. Mary's, the content is comprehensive and should be adaptable to other surgeries. It helps the reader to good understanding of the whole. Here are co-ordinated useful visual aids and guides for the nurse in her role as a member of the surgical team. Included are detailed descriptions of the operative procedure, positioning, draping and instruments. These units include sketches and photographs showing the application of instruments and equipment to the procedure. It touches briefly upon the basic program of learning and practice for the student nurse in this branch of nursing, also upon the responsibilities of the graduate nurse in her capacity of teacher, supervisor, and assistant.

E. W. S.

Neomycin. Nature, Formation, Isolation and Practical Application. By SELMAN A. WAKSMAN. 219 pages. $4.00. Rutgers University Press. New Brunswick, New Jersey.

In this monograph, the author traces the development of Neomycin from the time it is screened from one of thousands of isolated colonies of Actinomycetes until it reached the

stage of clinical trial. In contrast to much of the literature about antibiotics, the first 147 pages describe completely the discovery of Neomycin, its isolation, purification, and production, its physical, chemical and biological properties. Even production and engineering problems unfold in lucid and fascinating detail. The author proceeds to clinical studies in the remaining 50 pages. Nearly every question that comes to mind concerning a relatively new drug is answered. Some examples are the problems of bacteriostatic versus bacteriocidal properties, synergistic potentialities, and anti-tubercular activity.

In addition to its use as a reference where all the information to date on Neomycin is at one's fingertips, the book will serve another function. This story of the development of a drug is an excellent illustration of the modern philosophy of experimental science, the philosophy that a single investigator working in a poorly equipped laboratory cannot make an important contribution to science. Proponents of this idea maintain that only in magnificently equipped laboratories employing the collaboration of scientific specialists can significant advances be made. This reader does not subscribe to that belief, but concedes that the book presents an excellent illustration of this concept in the field of antibiotics.

Neomycin has use limited to topical application, intestinal antisepsis, and anti-tubercular therapy. Perhaps this book will stimulate volumes of similar excellence on the more commonly employed antibiotics.

HENRY N. WAGNER, JR.

BOOKS RECEIVED FOR REVIEW

Clinical Obstetrics. By MEMBERS OF THE STAFF OF THE PENNSYLVANIA HOSPITAL. Edited by CLIFFORD B. LULL AND ROBERT A. KIMBROUGH. 750 pp., $10.00. J. B. Lippincott Co., Philadelphia, Pa.

Die Körperlichen Behandlungsverfahren in der Psychiatrie. Vol. 1: Die Insulinbehandlung. By Max Muller. 295 pp., Ganzl. DM 36. Georg Thieme Verlag, Stuttgart, Germany.

Die Psychiatrie des Morbus Addison insbesondere seiner chronischen Forman. (Sammlung psychiatrischer und neurologischer Einzeldarstellungen). By W. A. STOLL. 144 pp., Kart. DM 18. Georg Thieme Verlag, Stuttgart, Germany.

Einführung in die Enzephalographie (Pneumenzephalographie). By OTTO SCHIERSMANN. 135 pp., Ganzl. DM 36. Georg Thieme Verlag, Stuttgart, Germany.

Endocrine Treatment in General Practice. Edited by MAX A. GOLDZIEHER AND JOSEPH W. GOLDZIEHER. 488 pp., $8.00. Springer Publishing Co. Inc., New York.

Handbook of Orthopaedic Surgery, 4th edition. By ALFRED RIVES SHANDS, JR. In Collaboration With Richard Beverly Raney. 644 pp., $8.00. The C. V. Mosby Co., St. Louis, Missouri.

Nurses Handbook of Obstetrics, 9th edition. By LOUISE ZABRISKIE AND N. J. EASTMAN. 695 pp., $4.75. J. B. Lippincott Co., Philadelphia, Pa.

Our Common Neurosis. Notes on a Group Experiment. By CHARLES B. THOMPSON AND ALFREDA P. SILL. Foreword by Trigant Burrow. 210 pp., $3.50. Exposition Press, New York.

Poliomyelitis. Papers and Discussions Presented at the Second International Poliomyelitis Conference. Compiled and Edited for the International Poliomyelitis Congress. 555 pp., $7.50. J. B. Lippincott Co., Philadelphia, Pa.

The Radiant Universe. By GEORGE W. HILL. 489 pp., $4.75. Philosophical Library, New York.

Taschenbuch der Praktischen Medizin. By JOHANNES KOTTMAIER. 937 pp., Ganzl. DM 29.70. Georg Thieme Verlag, Stuttgart, Germany.

Therapeutische Technik für die ärztiche Praxis. By KARL HANSEN. 985 pp., Ganzl. DM 89. Georg Thieme Verlag, Stuttgart, Germany.

Visual Anatomy: Thorax and Abdomen. By SYDNEY M. FRIEDMAN. 212 pp., $10.50. Charles C Thomas, Springfield, Illinois.